

PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau



INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

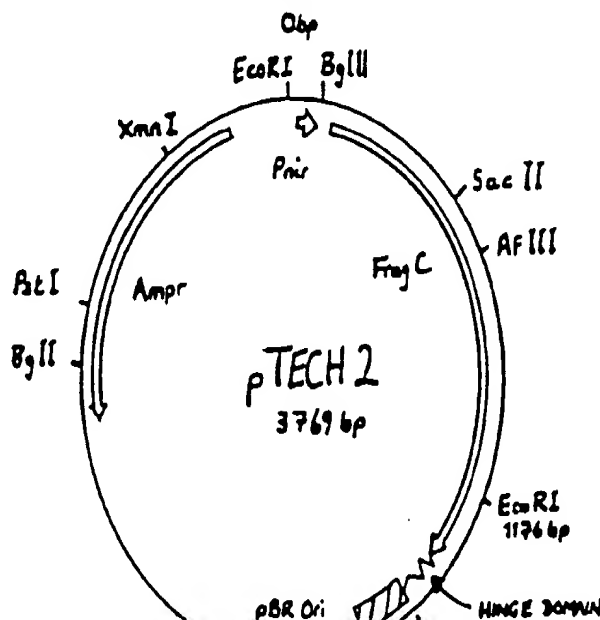
<p>(51) International Patent Classification⁵ : C12N 15/62, 15/31, 15/54 C12N 1/21, C07K 13/00 A61K 39/08 // (C12N 1/21 C12R 1:42)</p>	<p>A1</p>	<p>(11) International Publication Number: WO 94/03615 (43) International Publication Date: 17 February 1994 (17.02.94)</p>
<p>(21) International Application Number: PCT/GB93/01617 (22) International Filing Date: 30 July 1993 (30.07.93) (30) Priority data: 9216317.9 31 July 1992 (31.07.92) GB 9306398.0 26 March 1993 (26.03.93) GB (71) Applicant (for all designated States except US): MEDEVA HOLDINGS B.V. [NL/NL]; Churchill-Laan 223, NL-1078 ED Amsterdam (NL). (72) Inventors; and (75) Inventors/Applicants (for US only) : KHAN, Mohammed, Anjam [GB/GB]; HORMAECHE, Carlos, Estenio [GB/GB]; VILLARREAL-RAMOS, Bernardo [MX/GB]; Cambridge University Department of Pathology, Tennis Court Road, Cambridge CB2 1QP (GB). CHATFIELD, Steven, Neville [GB/GB]; Medeva Vaccine Research Unit, Department of Biochemistry, Imperial College of Science & Technology, London SW7 2AY (GB). DOUGAN, Gordon [GB/GB]; Department of Biochemistry, Imperial College of Science & Technology, London SW7 2AY (GB).</p>		<p>(74) Agents: HUTCHINS, Michael, Richard et al.; Fry Heath & Spence, St. Georges House, 6 Yattendon Road, Horley, Surrey RH6 7BS (GB) et al. (81) Designated States: AT, AU, BB, BG, BR, BY, CA, CH, CZ, DE, DK, ES, FI, GB, HU, JP, KP, KR, KZ, LK, LU, MG, MN, MW, NL, NO, NZ, PL, PT, RO, RU, SD, SE, SK, UA, US, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG). Published With international search report. With an indication in relation to a deposited microorganism furnished under Rule 13bis separately from the description. Date of receipt by the International Bureau: 13 October 1993 (13.10.93)</p>

71

(54) Title: EXPRESSION OF RECOMBINANT FUSION PROTEINS IN ATTENUATED BACTERIA

(57) Abstract

The invention provides a DNA molecule comprising a promoter sequence operably linked to a DNA sequence encoding first and second proteins linked by a hinge region wherein in that the promoter sequence can be one having activity which is induced in response to a change in the surrounding environment and the first protein can be Tetanus toxin C fragment or one or more



FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	FR	France	MR	Mauritania
AU	Australia	GA	Gabon	MW	Malawi
BB	Barbados	GB	United Kingdom	NE	Niger
BE	Belgium	GN	Guinea	NL	Netherlands
BF	Burkina Faso	GR	Greece	NO	Norway
BG	Bulgaria	HU	Hungary	NZ	New Zealand
BJ	Benin	IE	Ireland	PL	Poland
BR	Brazil	IT	Italy	PT	Portugal
BY	Belarus	JP	Japan	RO	Romania
CA	Canada	KP	Democratic People's Republic of Korea	RU	Russian Federation
CF	Central African Republic	KR	Republic of Korea	SD	Sudan
CG	Congo	KZ	Kazakhstan	SE	Sweden
CH	Switzerland	LI	Liechtenstein	SI	Slovenia
CI	Côte d'Ivoire	LK	Sri Lanka	SK	Slovak Republic
CM	Cameroon	LU	Luxembourg	SN	Senegal
CN	China	LV	Latvia	TD	Chad
CS	Czechoslovakia	MC	Monaco	TC	Togo
CZ	Czech Republic	MG	Madagascar	UA	Ukraine
DE	Germany	ML	Mali	US	United States of America
DK	Denmark	MN	Mongolia	UZ	Uzbekistan
ES	Spain			VN	Viet Nam
FI	Finland				

EXPRESSION OF RECOMBINANT FUSION PROTEINS IN ATTENUATED BACTERIA

This invention relates to DNA constructs, replicable expression vectors containing the constructs, attenuated bacteria containing the constructs and vaccines containing the said bacteria.

In recent years, there has emerged a new generation of live oral salmonella vaccines based upon strains of Salmonella which have been attenuated by the introduction of a non-reverting mutation in a gene in the aromatic biosynthetic pathway of the bacterium. Such strains are disclosed, for example, in EP-A-0322237. The aforesaid live oral salmonella vaccines are showing promise as vaccines for salmonellosis in man and animals, and they can also be used effectively as carriers for the delivery of heterologous antigens to the immune system. Combined salmonella vaccines have been used to deliver antigens from viruses, bacteria, and parasites, eliciting secretory, humoral and cell-mediated immune responses to the recombinant antigens. Combined salmonella vaccines show great potential as single dose oral multivaccine delivery systems [C. Hormaeche *et al*, FEMS Symposium No. 63, Plenum,

New York; pp 71-83, 1992].

There are problems to be overcome in the development of combined salmonella vaccines. A major consideration is obtaining a high level of expression of the recombinant antigen in the salmonella vaccine so that it will be sufficient to trigger an immune response. However, unregulated high level expression of foreign antigens can be toxic and affect cell viability [I. Charles and G. Dougan, TIBTECH 8, pp 117-21, 1990], rendering the vaccine ineffective or causing loss of the recombinant DNA. Several possible solutions to this problem have been described, such as expression from plasmids carrying essential genes, "on-off" promoters or incorporation of the foreign genes into the salmonella chromosome.

An alternative approach to overcoming the aforesaid problem would be to use a promoter which is inducible in vivo, and one such promoter is the E.coli nitrite reductase promoter nirB which is induced under anaerobiosis and has been used in biotechnology for the production of tetanus toxin fragment C (TetC) of Clostridium tetani [M.D. Oxeer et al Nucl. Ac. Res., 19, pp 2889-92, 1991]. It has previously been found by the inventors of this application (S.N. Chatfield et al Bio/Technology, Vol. 10, pp 888-92 1992) that an Aro Salmonella harbouring a construct expressing TetC from the nirB promoter (pTETnir15) elicited very high anti-tetanus antibody responses in mice. The article by Chatfield et al was published after the priority date of this application.

However, we have also found that when it was attempted to express the P28 antigen from Schistosoma mansoni alone from nirB, the resulting construct was not immunogenic.

Tetanus toxoid has been extensively used as an adjuvant for chemically coupled guest epitopes [D.A. Herrington et al. Nature, 328, pp 257-9 1987]. The potent immunogenicity of TetC in Salmonella suggested to us that it may be possible to exploit this character to promote the immune response of the guest peptides or proteins. However, fusing two proteins together often leads to an incorrectly folded chimaeric protein which no longer retains the properties of the individual components. For example the B subunit of the Vibrio cholerae (CT-B) and E.coli (LT-B) enterotoxins are powerful mucosal immunogens but genetic fusions to these subunits can alter the structure and properties of the carrier and hence their immunogenicity [see M. Sandkvist et al. J. Bacteriol 169, pp 4570-6, 1987, Clements 1990 and M. Lipscombe et al [Mol. Microbiol 5, pp 1385 1990]. Moreover, many heterologous genes expressed in bacteria are not produced in soluble properly folded, or active forms and tend to accumulate as insoluble aggregates [see C. Schein et al. Bio/Technology 6, pp 291-4, 1988 and R. Halenbeck et al; Bio/Technology 7, pp 710-5, 1989].

It is an object of the invention to overcome the aforementioned problems.

We have now found that efficient expression of recombinant antigens, and in particular fusion proteins,

can be achieved in bacteria such as salmonellae, by the use of an inducible promoter such as nirB and by incorporating a flexible hinge region between two antigenic components of the fusion protein. The resulting recombinant antigens have been shown to have good immunogenicity. It has also been found, surprisingly, that enhanced expression of a protein can be obtained when a gene coding for the protein is linked to the gene for tetanus toxin C fragment.

Accordingly, in a first aspect, the present invention provides a DNA construct comprising a promoter sequence operably linked to a DNA sequence encoding first and second proteins linked by a hinge region, characterised in that the promoter sequence is one having activity which is induced in response to a change in the surrounding environment.

In another aspect, the invention provides a DNA construct comprising a promoter sequence operably linked to a DNA sequence encoding linked first and second proteins, wherein the first heterologous protein is an antigenic sequence comprising tetanus toxin fragment C or one or more epitopes thereof.

In a further aspect, the invention provides a replicable expression vector, suitable for use in bacteria, containing a DNA construct as hereinbefore defined.

In a another aspect, the invention provides a fusion protein, preferably in substantially pure form, the fusion protein comprising linked (e.g. by a hinge region) first and second proteins, the fusion protein being expressed by

a replicable expression vector as hereinbefore defined.

In a further aspect the invention provides a process for the preparation of an attenuated bacterium which comprises transforming an attenuated bacterium with a DNA construct as hereinbefore defined.

The invention also provides a vaccine composition comprising an attenuated bacterium, or a fusion protein, as hereinbefore defined, and a pharmaceutically acceptable carrier.

The first and second proteins are preferably heterologous proteins and in particular can be polypeptide immunogens; for example they may be antigenic sequences derived from a virus, bacterium, fungus, yeast or parasite. In particular, it is preferred that the first said protein is an antigenic sequence comprising tetanus toxin fragment C or epitopes thereof.

The second protein is preferably an antigenic determinant of a pathogenic organism. For example, the antigenic determinant may be an antigenic sequence derived from a virus, bacterium, fungus, yeast or parasite.

Examples of viral antigenic sequences for the first and/or second heterologous proteins are sequences derived from a type of human immuno-deficiency virus (HIV) such as HIV-1 or HIV-2, the CD4 receptor binding site from HIV, for example from HIV-1 or -2., hepatitis A or B virus, human rhinovirus such as type 2 or type 14, Herpes simplex virus, poliovirus type 2 or 3, foot-and-mouth

virus, coxsackie virus, human papilloma virus (HPV), for example the type 16 papilloma virus, the E7 protein thereof, and fragments containing the E7 protein or its epitopes; and simian immunodeficiency virus (SIV).

Examples of antigens derived from bacteria are those derived from Bordetella pertussis (e.g. P69 protein and filamentous haemagglutinin (FHA) antigens), Vibrio cholerae, Bacillus anthracis, and E.coli antigens such as E.coli heat Labile toxin B subunit (LT-B), E.coli K88 antigens, and enterotoxigenic E.coli antigens. Other examples of antigens include the cell surface antigen CD4, Schistosoma mansoni P28 glutathione S-transferase antigens (P28 antigens) and antigens of flukes, mycoplasma, roundworms, tapeworms, Chlamydia trachomatis, and malaria parasites, eg. parasites of the genus plasmodium or babesia, for example Plasmodium falciparum, and peptides encoding immunogenic epitopes from the aforementioned antigens.

Particular antigens include the full length Schistosoma mansoni P28, and oligomers (e.g. 2, 4 and 8-mers) of the immunogenic P28 aa 115-131 peptide (which contains both a B and T cell epitope), and human papilloma virus E7 protein, Herpes simplex antigens, foot and mouth disease virus antigens and simian immunodeficiency virus antigens.

The promoter sequence is one having activity which is induced in response to a change in the surrounding environment, and an example of such a promoter sequence is

one which has activity which is induced by anaerobic conditions. A particular example of such a promoter sequence is the nirB promoter which has been described, for example in International Patent Application PCT/GB92/00387. The nirB promoter has been isolated from E.coli, where it directs expression of an operon which includes the nitrite reductase gene nirB (Jayaraman et al, J. Mol. Biol. 196, 781-788, 1987), and nirD, nirC, cysG (Peakman et al, Eur. J. Biochem. 191, 315-323, 1990). It is regulated both by nitrite and by changes in the oxygen tension of the environment, becoming active when deprived of oxygen, (Cole, Biochem, Biophys. Acta. 162, 356-368, 1968). Response to anaerobiosis is mediated through the protein FNR, acting as a transcriptional activator, in a mechanism common to many anaerobic respiratory genes. By deletion and mutational analysis the part of the promoter which responds solely to anaerobiosis has been isolated and by comparison with other anaerobically-regulated promoters a consensus FNR-binding site has been identified (Bell et al, Nucl, Acids. Res. 17, 3865-3874, 1989; Jayaraman et al, Nucl, Acids, Res. 17, 135-145, 1989). It has also been shown that the distance between the putative FNR-binding site and the -10 homology region is critical (Bell et al, Molec. Microbiol. 4, 1753-1763, 1990). It is therefore preferred to use only that part of the nirB promoter which responds solely to anaerobiosis. As used herein, references to the nirB promoter refer to the promoter itself or a part or

derivative thereof which is capable of promoting expression of a coding sequence under anaerobic conditions. The preferred sequence, and which contains the nirB promoter is: AATTCAGGTAAATTTGATGTACATCAAATGGTACCCCTTGCTGAATCGTTAAGG TAGGCGGTAGGGCC (SEQ ID NO: 1)

The hinge region is a region designed to promote the independent folding of both the first and second proteins by providing both spatial and temporal separation between the domains.

The hinge region typically is a sequence encoding a high proportion of proline and/or glycine amino acids. The hinge region may be composed entirely of proline and/or glycine amino acids. The hinge region may comprise one or more glycine-proline dipeptide units.

The hinge region may, for example, contain up to about fifteen amino acids, for example at least 4 and preferably 6-14 amino acids, the number of amino acids being such as to impart flexibility between the first and second proteins.

In one embodiment, the hinge region can correspond substantially to the hinge domain of an antibody immunoglobulin. The hinge regions of IgG antibodies in particular are rich in prolines [T.E. Michaelson et al. J. Biol. Chem. 252, 883-9 1977], which are thought to provide a flexible joint between the antigen binding and tail domains.

Without wishing to be bound by any theory, the prolines are thought to form the rigid part of the hinge as

the ring structure characteristic of this amino acid hinders rotation around the peptide bond that connects the proline residue with an adjacent amino acid. This property is thought to prevent proline, and adjacent residues, from adopting the ordered structure of an alpha helix or beta strand. Flexibility is thought to be imparted by glycine, the simplest amino acid, with very limited steric demands. Glycine is thought to function as a flexible elbow in the hinge. Other amino acids may be substituted for glycine, particularly those without bulky side-chains, such as alanine, serine, asparagine and threonine.

In one preferred embodiment, the hinge region is a chain of four or more amino acids defining the sequence



wherein Pro is proline, X and Y are each glycine, or an amino acid having a non-bulky side chain; Z is any amino acid; p is a positive integer; q is a positive integer of from one to ten; and r is zero or a positive integer greater than zero.

The hinge region can be a discrete region heterologous to both the first and second proteins or can be defined by a carboxy-end portion of the first protein or an amino-end portion of the second protein.

Codons which are infrequently utilised in E.coli [H. Grosjean et al, Gene 18, 199-209, 1982] and Salmonella are selected to encode for the hinge, as such rare codons are thought to cause ribosomal pausing during translation of the messenger RNA and allow for the correct folding of

polypeptide domains [I.J. Purvis *et al.* J. Mol. Biol. 193, 413-7 1987]. In addition, where possible restriction enzymes are chosen for the cloning region which, when translated in the resulting fusion, do not encode for bulky or charged side-groups.

In a most preferred aspect, the present invention provides a DNA molecule comprising the nirB promoter operably linked to a DNA sequence encoding first and second polypeptide immunogens linked by a hinge region, wherein the first polypeptide immunogen comprises tetanus toxin fragment C or epitopes thereof.

In another preferred aspect of the invention, there is provided a replicable expression vector, suitable for use in bacteria, containing the nirB promoter sequence operably linked to a DNA sequence encoding first and second polypeptide immunogens linked by a hinge region, wherein the first polypeptide immunogen comprises tetanus toxin fragment C or epitopes thereof.

It has been found that by providing a DNA sequence encoding tetanus toxin fragment C (TetC) linked via a hinge region to a second sequence encoding an antigen, the expression of the sequence in bacterial cells is enhanced relative to constructs wherein the fragment C and hinge region are absent. For example, the expression level of the full length P28 protein of S. mansoni when expressed as a fusion to TetC was greater than when the P28 protein was expressed alone from the nirB promoter. The TetC fusions to the full length P28 protein of S. mansoni and its tandem

epitopes were all soluble and expressed in both E.coli and S.typhimurium. In addition, the TetC-P28 fusion protein was capable of being affinity purified by a glutathione agarose matrix, suggesting that the P28 had folded correctly to adopt a conformation still capable of binding to its natural substrate.

Stable expression of the first and second heterologous proteins linked by the hinge region can be obtained in vivo. The heterologous proteins can be expressed in an attenuated bacterium which can thus be used as a vaccine.

The attenuated bacterium may be selected from the genera Salmonella, Bordetella, Vibrio, Haemophilus, Neisseria and Yersinia. Alternatively, the attenuated bacterium may be an attenuated strain of enterotoxigenic Escherichia coli. In particular the following species can be mentioned: S.typhi - the cause of human typhoid; S.typhimurium - the cause of salmonellosis in several animal species; S.enteritidis - a cause of food poisoning in humans; S.choleraesuis - a cause of salmonellosis in pigs; Bordetella pertussis - the cause of whooping cough; Haemophilus influenzae - a cause of meningitis; Neisseria gonorrhoeae - the cause of gonorrhoea; and Yersinia - a cause of food poisoning.

Attenuation of the bacterium may be attributable to a non-reverting mutation in a gene in the aromatic amino acid biosynthetic pathway of the bacterium. There are at least

biosynthetic pathway. Several of these map at widely differing locations on the bacterial genome, for example aroA (5-enolpyruvylshikimate-3-phosphate synthase), aroC (chorismate synthase), aroD (3-dihydroquinate dehydratase) and aroE (shikimate dehydrogenase). A mutation may therefore occur in the aroA, aroC, aroD, or aroE gene.

Preferably, however, an attenuated bacterium harbours a non-reverting mutation in each of two discrete genes in its aromatic amino acid biosynthetic pathway. Such bacteria are disclosed in EP-A-0322237. Double aro mutants which are suitable are aroA aroC, aroA aroD, and aroA aroE. Other bacteria having mutations in other combinations of the aroA, aroC, aroD and aroE genes are however useful. Particularly preferred are Salmonella double aro mutants, for example double aro mutants of S.typhi or S.typhimurium, in particular aroA aroC, aroA aroD and aroA aroE mutants. Alternatively, the attenuated bacterium may harbour a non-reverting mutation in a gene concerned with the regulation of one or more other genes (EP-A-0400958). Preferably the mutation occurs in the ompR gene or another gene involved in regulation. There are a large number of other genes which are concerned with regulation and are known to respond to environmental stimuli (Ronson et al, Cell 49, 579-581).

This type of attenuated bacterium may harbour a second mutation in a second gene. Preferably the second gene is a gene encoding for an enzyme involved in an essential biosynthetic pathway, in particular genes

involved in the pre-chlorismate pathway involved in the biosynthesis of aromatic compounds. The second mutation is therefore preferably in the aroA, aroC or aroD gene.

Another type of attenuated bacterium is one in which attenuation is brought about by the presence of a non-reverting mutation in DNA of the bacterium which encodes, or which regulates the expression of DNA encoding, a protein that is produced in response to environmental stress. Such bacteria are disclosed in WO 91/15572. The non-reverting mutation may be a deletion, insertion, inversion or substitution. A deletion mutation may be generated using a transposon.

An attenuated bacterium containing a DNA construct according to the invention can be used as a vaccine. Fusion proteins (preferably in substantially pure form) expressed by the bacteria can also be used in the preparation of vaccines. For example, a purified TetC-P28 fusion protein has been found to be immunogenic on its own. In a further aspect therefore, the invention provides a vaccine composition comprising a pharmaceutically acceptable carrier or diluent and, as active ingredient, an attenuated bacterium or fusion protein as hereinbefore defined.

The vaccine may comprise one or more suitable adjuvants.

The vaccine is advantageously presented in a lyophilised form, for example in a capsular form, for oral administration to a patient. Such capsules may be provided

with an enteric coating comprising, for example, Eudragit "S", Eudragit "L", Cellulose acetate, Cellulose acetate phthalate or Hydroxypropylmethyl Cellulose. These capsules may be used as such, or alternatively, the lyophilised material may be reconstituted prior to administration, e.g. as a suspension. Reconstitution is advantageously effected in buffer at a suitable pH to ensure the viability of the organisms. In order to protect the attenuated bacteria and the vaccine from gastric acidity, a sodium bicarbonate preparation is advantageously administered before each administration of the vaccine. Alternatively, the vaccine may be prepared for parenteral administration, intranasal administration or intramammary administration.

The attenuated bacterium containing the DNA construct of the invention may be used in the prophylactic treatment of a host, particularly a human host but also possibly an animal host. An infection caused by a micro-organism, especially a pathogen, may therefore be prevented by administering an effective dose of an attenuated bacterium according to the invention. The bacterium then expresses a heterologous protein or proteins capable of raising antibody to the micro-organism. The dosage employed will be dependent on various factors including the size and weight of the host, the type of vaccine formulated and the nature of the heterologous protein.

An attenuated bacterium according to the present invention may be prepared by transforming an attenuated

bacterium with a DNA construct as hereinbefore defined. Any suitable transformation technique may be employed, such as electroporation. In this way, an attenuated bacterium capable of expressing a protein or proteins heterologous to the bacterium may be obtained. A culture of the attenuated bacterium may be grown under aerobic conditions. A sufficient amount of the bacterium is thus prepared for formulation as a vaccine, with minimal expression of the heterologous protein occurring.

The DNA construct may be a replicable expression vector comprising the nirB promoter operably linked to a DNA sequence encoding the tetanus toxin C fragment or epitopes thereof and the second heterologous protein, linked by a hinge region. The nirB promoter may be inserted in an expression vector, which already incorporates a gene encoding one of the heterologous proteins (e.g. tetanus toxin C fragment), in place of the existing promoter controlling expression of the protein. The hinge region and gene encoding the second heterologous protein (e.g. an antigenic sequence) may then be inserted. The expression vector should, of course, be compatible with the attenuated bacterium into which the vector is to be inserted.

The expression vector is provided with appropriate transcriptional and translational control elements including, besides the nirB promoter, a transcriptional termination site and translational start and stop codons. An appropriate ribosome binding site is provided. The

vector typically comprises an origin of replication and, if desired, a selectable marker gene such as an antibiotic resistance gene. The vector may be a plasmid.

The invention will now be illustrated but not limited, by reference to the following examples and the accompanying drawings, in which:

Figure 1 is a schematic illustration of the construction of an intermediate plasmid pTECH1 in accordance with one aspect of the invention.

Figure 2 is a schematic illustration of the construction of a second intermediate plasmid pTECH2.

Figure 3 is a schematic illustration of the construction of a plasmid of the invention using the intermediate plasmid of Figure 2 as the starting material. In Figure 3 B = BamHI, E = EcoRV; H = HindIII; X = XbaI; S = SpeI.

Figure 4 is a schematic illustration of the construction of a plasmid containing repeating epitopes (repiteps).

Figure 5 illustrates antibody responses against recombinant S. mansoni protein P28 as detected by ELISA in mice inoculated intravenously with SL3261, SL3261(pTETnir15), SL3261(pTECH2), SL3261(pTECH2-monomer), SL3261 (pTECH2-dimer), SL3261(pTECH2-tetramer), SL3261(pTECH2-octamer), and SL3261(pTECH1-P28). In Figure 5 the results are expressed as OD in individual mice at intervals after immunisation.

Figure 6 illustrates antibody responses against TetC

as detected by ELISA in mice inoculated as in Figure 5.

Figure 7 illustrates antibody responses against peptide 115-131 of the P28 protein coupled to ovalbumin as detected by ELISA in mice inoculated intravenously with SL3261, SL3261(pTECH2), SL3261(pTECH2-monomer), SL3261(pTECH2-dimer), SL3261(pTECH2-tetramer), and SL3261(pTECH2-octamer).

Figure 8 illustrates antibody responses against TetC as detected by ELISA from mice inoculated orally with SL3261(pTECH1-P28).

Figure 9 illustrates antibody responses against recombinant P28 as detected by ELISA in mice inoculated as in Figure 8.

Figure 10 illustrates schematically the preparation of various constructs from the pTECH2 intermediate plasmid.

Figure 11 illustrates schematically the structure of tripartite protein structures ("heteromers") prepared using pTECH2.

Figure 12 shows the DNA sequence of the vector pTECH1. (SEQ ID NO: 17).

Figure 13 shows the DNA sequence of the vector pTECH2. (SEQ ID NO: 18).

Figure 14 illustrates, schemetically, the restriction sites on the vector pTECH2.

EXAMPLE 1

Preparation of pTECH1

The preparation of pTECH1, a plasmid incorporating the

nirB promoter and TetC gene, and a DNA sequence encoding a hinge region and containing restriction endonuclease sites to allow insertion of a gene coding for a second or guest protein, is illustrated in Figure 1. Expression plasmid pTETnir15, the starting material shown in Figure 1, was constructed from pTETtac115 (Makoff et al, Nucl. Acids Res. 17 10191-10202, 1989); by replacing the EcoRI-ApaI region (1354bp) containing the lacI gene and tac promoter with the following pair of oligos 1 and 2:

Oligo-1 5'AATTCAGGTAAATTTGATGTACATCAAATGGTACCCCTTGCTGAAT

Oligo-2 3'-GTCCATTTAAACTACATGTAGTTTACCATGGGGAACGACTTA

CGTTAAGGTAGGCGGTAGGGCC-3' (SEQ ID NO: 2)

GCAATTCATCCGCCATC-5' (SEQ ID NO: 3)

The oligonucleotides were synthesised on a Pharmacia Gene Assembler and the resulting plasmids confirmed by sequencing (Makoff et al, Bio/Technology 7, 1043-1046, 1989).

The pTETnir15 plasmid was then used for construction of the novel pTECH1 plasmid incorporating a polylinker region suitable as a site for insertion of heterologous DNA to direct the expression of fragment C fusion proteins. pTETnir15 is a known pAT153-based plasmid which directs the expression of fragment C. However, there are no naturally occurring convenient restriction sites present at the 3'-end of the TetC gene. Therefore, target sites, preceded by a hinge region, were introduced at the 3'-end of the TetC coding region by means of primers tailored with "add-on" adapter sequences (Table 1), using the polymerase

chain reaction (PCR) [K. Mullis et al, Cold Spring Harbor Sym. Quant. Biol. 51, 263-273 1986]. Accordingly, pTETnir15 was used as a template in a PCR reaction using primers corresponding to regions covering the SacII and BamHI sites. The anti-sense primer in this amplification was tailored with a 38 base 5'-adaptor sequence. The anti-sense primer was designed so that a sequence encoding novel XbaI, SpeI and BamHI sites were incorporated into the PCR product. In addition, DNA sequences encoding additional extra amino acids including proline were incorporated (the hinge regions) and a translation stop codon signal in frame with the fragment C open reading frame.

The PCR product was gel-purified and digested with SacII and BamHI, and cloned into the residual 2.8 kb vector pTETnir15 which had previously been digested by SacII and BamHI. The resulting plasmid purified from transformed colonies and named pTECH 1 is shown in Figure 1.

Heterologous sequences such as the sequence encoding the

multiple tandem epitopes, ("repiteps") (Figure 2). Two complementary oligonucleotides were synthesised bearing the restriction enzyme target sites for BamHI, EcoRV, HindIII, SpeI, followed by a translational stop codon (Table 1). The oligonucleotides were tailored with XbaI and BamHI cohesive ends; however, the BamHI target sequence was designed to include a mismatch and, upon cloning, this restriction site in pTECH1 is destroyed. This version of the vector was designated pTECH2.

EXAMPLE 3

Construction of pTECH1-P28

A P28 gene expression cassette was produced by PCR using pUC19-P28 DNA (a kind gift from Dr R Pierce, Pasteur Institute, Lille) as template. Oligonucleotide primers were designed to amplify the full length P28 gene beginning with the start codon and terminating with the stop codon. In addition, the sense and antisense primers were tailored with the restriction sites for XbaI and BamHI respectively. The product was gel-purified and digested with XbaI and BamHI and then cloned into pTECH1 which had previously been digested with these enzymes and subsequently gel-purified.

Expression of the TetC-P28 fusion protein

Expression of the TetC-P28 fusion protein was evaluated by SDS-PAGE and Western blotting of bacterial cells harbouring the construct. It was found that the fusion protein remains soluble, cross-reacts with antisera to both TetC and P28, and is also of the expected molecular weight.

The fusion protein was stably expressed in a number of different genetic backgrounds including E.coli (TG2) and S. typhimurium (SL5338,SL3261) as judged by SDS-PAGE and Western blotting. Of interest was a minor band of 50kDal which co-migrates with the TetC-Hinge protein alone and cross-reacts exclusively with the anti-TetC sera is visible in a Western blot. As the codon selection in the hinge region has been designed to be suboptimal, the rare codons may cause pauses during translation which may occasionally lead to the premature termination of translation, thus accounting for this band.

Affinity purification of the TetC-P28 fusion

Glutathione is the natural substrate for P28, a glutathione S-transferase. The amino acid residues involved in binding glutathione are thought to be spatially separated in the primary structure of the polypeptide and brought together to form a glutathione binding pocket in the tertiary structure (P. Reinemer *et al.* EMBO, J8, 1997-2005, 1991). In order to gauge whether the P28 component of the fusion has folded correctly to adopt a conformation capable of binding glutathione, its ability to be affinity purified on a glutathione-agarose matrix was tested. The results obtained (not shown) demonstrated that TetC-P28 can indeed bind to the matrix and the binding is reversible, as the fusion can be competitively eluted with free glutathione.

EXAMPLE 4

Construction of pTECH2-P28(aal15-131) peptide fusions

Complementary oligonucleotides encoding the aal15-131 peptide were designed with a codon selection for optimal expression in E.coli [H. Grosjean et al idem]. The oligonucleotides were tailored with BglIII and SpeI cohesive ends which were generated upon annealing and cloned into pTECH2 which had previously been digested with BamHI and SpeI (Figure 3).

Repeated tandem copies of the epitopes (repitopes) were constructed in pTECH2 by the following approach. The recombinant fusion vector was digested with XbaI and SpeI and to each digest was added a second restriction enzyme which cuts uniquely elsewhere within the vector, e.g. PstI which makes a cut exclusively within the ampicillin resistance gene (Figure 4). DNA fragments containing the epitope sequences can be purified from each of the double digests, mixed and then ligated. XbaI cleaves its target sequence to generate a 5'-CTAG overhang which is compatible with the SpeI overhang. Upon ligation the recognition sequences of both these enzymes are destroyed. In this way the XbaI-SpeI restriction sites remain unique and the procedure can be simply and effectively repeated to construct recombinant fusion vectors expressing four or eight tandem copies of the epitopes (Figure 4). A similar strategy has been used by others in the generation of a multimeric fusion protein for the production of a neuropeptide [T. Kempe et al. Gene 39, 239-45, 1985].

Expression of the TetC-peptide fusion proteins

Expression of the TetC-peptide fusions as monomeric,

dimeric, tetrameric, and octameric tandem peptide repeats was evaluated by SDS-PAGE and Western blotting of the bacterial strains harbouring the constructs. The fusion proteins remain soluble, cross-react with both antisera to TetC and P28, and are also of the expected molecular weight [Figure 5]. Furthermore the fusion proteins are expressed in a number of different genetic backgrounds including E.coli (TG2) and S. typhimurium (SL5338, SL3261) as judged by SDS-PAGE and Western blotting. There appeared to be some degradation of the epitopes consisting of higher numbers of copies, as indicated by the appearance of faint bands of lower molecular weight seen in Western blots probed with the anti-P28 antibody.

The size of the bands suggested that they consisted of reduced copy number fusions to TetC. As was the case with the TetC-P28 fusion described above, the level of expression of the TetC-peptide fusions was less than that of TetC alone from pTECH2, with the expression level gradually decreasing with increasing copy number.

EXAMPLE 5

Immunological Studies

Stability of the plasmid constructs in vivo and immunisation of mice

BALB/c mice were given approx. 10^6 cfu i/v or 5×10^9 orally of S. typhimurium SL3261 and SL3261 harbouring the different constructs. Viable counts on homogenates of liver, spleen and (for orally inoculated mice) lymph nodes performed from days 1-8 (epitope fusions) and 1-11 (vector,

octamer and P28 fusions) were similar on media with and without ampicillin, indicating that the plasmids were not being lost during growth in the tissues.

Antibody responses in mice immunised intravenously

Antibody responses to the TetC-P28 fusion

Tail bleeds were taken weekly on weeks 3 to 6 from animals from each group of 8 mice. Figure 5 shows that in mice immunised with salmonellae expressing the TetC-P28 fusion, antibody responses to recombinant P28 appeared by week 3, and were positive in 6/6 mice from week 4 onwards. No anti-P28 antibodies were detected in sera from mice immunised with either SL3261 or SL3261-pTETnir15 or pTECH2.

All mice immunised with salmonellae expressing TetC, either alone or as the TetC-P28 fusion (but not with salmonellae alone), made antibody to TetC appearing as

mice immunised either with SL3261, pTECH2 or the monomeric epitope fusion.

Some of the anti-epitope sera recognised the full length P28 protein in ELISA (Figure 5). One mouse injected with the dimeric fusion was positive at week 5, another mouse injected with the tetrameric fusion was positive at week 3. Thereafter sera from at least two mice injected with the octameric fusion consistently recognised P28 from week four up to week six.

In summary the antibody responses against the repitopes improved dramatically with increasing copy number, with the tetrameric and octameric repitope fusions being the most potent. No antibody responses to the monomeric fusion were detected.

Antibody response to TetC in mice immunised with the different fusions

The antibody response to TetC was not the same in all groups; the addition of C-terminal fusions to TetC clearly modified the response. Figure 6 shows that the antibody response to TetC elicited by the vector pTECH2 (TetC-Hinge alone) was significantly less than the TetC response to the parental vector, pTETnir15. Surprisingly, the addition to TetC of fusions of increasing size dramatically restores the response to TetC. The anti-TetC response to the largest fusion, full length P28 in pTECH1, was similar to the response to TetC obtained from the parental plasmid (under the conditions tested). Sera from mice injected with non-recombinant SL3261 did not react with TetC at any

time during the period tested.

Antibody responses in mice immunised orally

Groups of 10 mice were immunised orally with approx. 5×10^9 cfu of SL3261 alone or carrying pTECH1, or pTECH1-P28, given intragastrically in 0.2ml via a gavage tube. Bleeds taken from week 3 to week 10 showed that most mice receiving the recombinant salmonellae made antibody to TetC as early as week 3 (Figure 8). Mice immunised with the TetC-P28 fusion made antibody to P28 which was detectable in approximately half of the mice by week 8, and then declined (Figure 9).

Antibody responses in mice immunised with the purified fusion protein

Mice were immunised subcutaneously with affinity purified TetC-P28 fusion protein adsorbed on aluminium hydroxide. Controls received commercial tetanus toxoid alone. Preliminary results indicate that animals given the fusion protein make an antibody response to both TetC and to P28 (data not shown). No anti-P28 antibody was detected in mice given tetanus toxoid.

T-cell responses to TetC and P28

Mice were immunised i/v with approximately 10^6 cfu of SL3261, SL3261(pTETnir15) and SL3261(pTECH1-P28). Six months later T-cell responses as IL-2/IL-4 production were measured against salmonella whole cell soluble extract,

TetC, recombinant P28 and whole adult worm antigen as described in the section headed Materials and Methods below. Table 2 shows that cells from both groups produced an IL-2/IL-4 response to the sodium hydroxide treated salmonella extract and to TetC. However, cells from mice immunised with the salmonellae expressing the TetC-P28 fusion also responded to both recombinant P28 and whole worm extract.

Thus the salmonella delivery system has elicited both humoral and cellular (T-cell) immune responses to P28.

The salmonellae expressing the recombinant antigens all persisted in the mouse tissues as well as the parental strain, and the plasmids were not lost in vivo.

Constructs expressing higher molecular weight fusions (full length P28 and octamer) proved to be the most immunogenic. It may be that the immune response has been promoted by the carrier TetC providing additional T-cell helper epitopes [Francis et al. Nature 330: 168-170, 1987]. By week 4 all the mice immunised with cells carrying pTECH1-P28 responded to both TetC and also the full length P28 protein following *in vivo* immunisation. Mice immunised

dramatically with increasing copy number, with the tetramer and octamer "repite" fusions displaying the greatest potency.

EXAMPLE 6

Cloning of HPVE7 protein in pTECH2

The full-length HPV type 16 E7 protein gene was cloned into plasmid pTECH2 by an in frame insertion of the gene in the BamHI site of the vector hinge region.

The E7 gene was obtained from plasmid pGEX16E7 (S.A. Comerford *et al.* J Virology, 65, 4681-90 1991). The gene in this plasmid is flanked by two restriction sites: a 3' BamHI site and a 5' EcoRI site. pGEX16E7 DNA was digested with EcoRI and blunt ended by a filling up reaction using Sequenase (DNA polymerase from USB). It was then digested with BamHI to release the 0.3 Kbp full length E7 gene.

The gel purified gene was ligated to BamHI-EcoRV double digested pTECH2 and this ligation mixture used to transform competent E.coli HB101 bacteria.

Recombinant colonies were selected by colony blotting using two monoclonal antibodies against HPV16 E7 protein as probes, namely 6D and 4F (R.W. Tindle, *et al* J Gen.Vir. 71,1347-54 1990). One of these colonies, named pTE79, was chosen for further analysis.

Protein extracts from pTE79 transformed E.coli grown in both aerobic and anaerobic conditions were prepared and analysed by SDS-PAGE and Western blotting. Growth in anaerobic conditions resulted in expression of a recombinant molecule of about 60 KDa which reacted with

monoclonal antibodies 6D and 4F and a rabbit polyclonal serum against Tetanus fragment C.

EXAMPLE 7

Construction of pTECH2-gD

An immunologically important antigen from herpes simplex virus type 1 [HSV1] is glycoprotein D, termed gD1 (R.J. Watson *et al* Science 218, 381-383 1982). A truncated gD1 gene cassette, lacking the transmembrane and cytoplasmic domains aa26-340, was synthesised by PCR. The PCR primers used are shown in Table 3. The forward primer was designed to encode the N-terminus of the mature protein and the reverse primer encoded the amino acids immediately 5' to the transmembrane domain. In addition the primers were tailored with BamHI and SpeI restriction sites respectively. The template for the PCR reaction was the plasmid pRWFG [a HSV1 gD BamHI-J clone from strain Patton in pBR322; a kind gift from Dr. T. Minson, Cambridge University]. The amplification product was digested with BamHI and SpeI and cloned into pTECH2 which had previously been digested with the respective enzymes.

Expression of the TetC-gD1 fusion protein was assessed by SDS-PAGE and Western blotting of bacterial strains harbouring the constructs. The Western blots were probed with either anti-TetC polyclonal sera or a monoclonal antibody directed against amino acids 11-19 of the mature gD [designated LP16, obtained from Dr. T. Minson, Cambridge]. The fusion protein is expressed as a 85kDal

weight bands down to 50kDal in size. The lower molecular weight bands could correspond to proteolytic cleavage products of gD or represent the products of premature translational termination within the coding region of gD due to ribosomal pausing. The fusion protein is expressed in the salmonella strains SL5338 and SL3261.

EXAMPLE 8

Construction of pTECH2- FMDV/SIV Repitopes

Peptides from the foot and mouth disease virus (FMDV; serotype A12] viral protein1 [VP1; aa136-159] and the V2 loop from simian immunodeficiency virus [SIV] envelope protein [gp120; aa171-190] were cloned into pTECH2 (M.P. Broekhuijsen et al J. Gen. Virol. 68, 3137-45 1987; K.A. Kent et al. AIDS Res. and Human Retro. 8:1147-1151 1992].

Complementary oligonucleotides encoding the peptides were designed with a codon selection for optimal expression in E. coli [H. Grosjean et al Gene, 18, 199-209, 1982]. The oligonucleotides are shown in Table 3. The oligonucleotides were tailored with BglII and SpeI cohesive ends which were generated upon annealing and cloned into pTECH2 which had previously been digested with BamHI and SpeI (Figure 3). Dimeric, tetrameric and octameric fusions of these peptides were constructed as described previously.

Expression of the TetC-fusions was assessed by SDS-PAGE and Western blotting with a polyclonal sera directed against TetC and monoclonal antibodies directed against either the FMDV or the SIV epitopes. The FMDV and SIV

repitope constructs expressed the TetC fusion proteins in both SL5338 and SL3261.

EXAMPLE 9

Construction of pTECH2- gp120-P28 Peptide Heteromers

To explore the possibility of delivering more than one type of epitope from a single molecule of TetC, fusions have been made with the P28 and SIV repitopes to produce a tripartite protein. This form of construction has been facilitated by the modular nature of the vector which allows the assembly of vector modules containing different repitopes. These "heteromers" express either tandem dimers or tetramers of the P28 and SIV repitopes. To investigate the effect of the position of a particular repitope in the TetC-Repitope A-Repitope B fusion on its expression level, stability, and immunogenicity, the converse combinations have also been constructed i.e. TetC-Repitope B-Repitope A, as is shown in Figure 11. "Heteromers" constructed in this way are TetC-P28 dimer-SIV dimer, TetC-SIV dimer-P28 dimer, TetC-P28 tetramer-SIV tetramer and TetC-SIV tetramer-P28 tetramer.

Expression of the tripartite fusions were evaluated by SDS-PAGE and Western blotting using the antibody reagents described above. These heteromer constructs are all expressed in the *Salmonella* strains SL5338 and SL3261, but intriguingly the expression level and stability is greater in one dimer-dimer and tetramer-tetramer combination (TetC-gp120-P28] than the converse.

EXAMPLE 10

MATERIALS AND METHODS

Plasmids, Oligonucleotides, and the Polymerase Chain Reaction

The plasmid pTETnir15 directs the expression of fragment C from tetanus toxin under the control of the nirB promoter [Chatfield *et al.* idem Ozer *et al.* idem]. The TetC-hinge fusion vector pTECH1 was constructed from pTETnir15 by the polymerase chain reaction (PCR) described by Mullis *et al.*, 1986. PCR was performed using the high-fidelity thermostable DNA polymerase from Pyrococcus furiosus, which possesses an associated 3'-5' exonuclease proofreading activity [K.S. Lundberg *et al.* Gene 108: 1-6, 1991]. The amplification reaction was performed according to the manufacturer's instructions (Stratagene).

Bacterial Strains

The bacterial strains used were E.coli TG2 (recA; [J. Sambrook *et al.* Molecular cloning: a laboratory manual. Cold Spring Harbor, New York, 1989]). S.typhimurium SL5338 (galE r⁺m⁺ [A. Brown J. Infect. Dis. 155: 86-92, *et al.* J. Infect. Dis. 155: 86-92, 1987]) and SL3261 (aroA); [S.K. Hoiseth *et al.* Nature 291, 238-9, 1981]. Bacteria were cultured in either L or YT broth and on L-agar with ampicillin (50 µg/ml) if appropriate. Plasmid DNA prepared in E.coli was first modified by transformation into SL5338 to increase the efficiency of electroporation into the SL3261 aroA (r⁺m⁺) vaccine. For electroporation, cells growing in mid-log phase were harvested and washed in half the initial culture volume of ice-cold water, 1/10 volume

of ice-cold glycerol (10%), and finally the cells were resuspended to a concentration of 10^{10} cells/ml in ice-cold glycerol (10%). To a pre-chilled cuvette was added a mix of 60 μ l cells and 100 ng of plasmid DNA. The cells were pulsed using the Porator from Invitrogen (settings: voltage=1750 μ v, capacitance = 40 μ F, resistance = 500). Prewarmed L-broth supplemented with 20 mM glucose was added immediately and the cells grown at 37°C with gentle shaking for 1-1.5 h. The cells were then plated on L-agar plates containing ampicillin and incubated at 37°C for 16 h.

SDS-PAGE and Western Blotting

Expression of the TetC fusions was tested by SDS-PAGE and Western blotting. Cells growing in mid-log phase with antibiotic selection were harvested by centrifugation and the proteins fractionated by 10% SDS-PAGE. The proteins were transferred to a nitrocellulose membrane by electroblotting and reacted with either a polyclonal rabbit antiserum directed against TetC or the full length P28 protein. The blots were then probed with goat anti-rabbit-Ig conjugated to horseradish peroxidase (Dako, UK) and developed with 4-chloro-1-naphthol.

Glutathione-Agarose Affinity Purification

Bacterial cells expressing the TetC full length P28 gene fusion were grown to log phase, chilled on ice, and harvested by centrifugation at 2500Xg for 15 min at 4°C. The cells were resuspended in 1/15th the original volume of ice-cold phosphate buffered saline (PBS) and lysed by sonication in a MSE Soniprep. The insoluble material was

removed by centrifugation and to the supernatant was added 1/6 volume of a 50% slurry of pre-swollen glutathione-agarose beads. (Sigma, UK.). After mixing gently at room temperature for 1 h the beads were collected by centrifugation at 1000Xg for 10 sec. The supernatant was discarded and the beads resuspended in 20 volumes of cold PBS-0.5% Triton X-100 and the beads collected again by centrifugation. The washing step was repeated three more times. The fusion protein was eluted by adding 1 volume of 50 mM Tris-HCl, pH 8.0 containing 5.0 mM reduced glutathione (Sigma). After mixing gently for 10 min the beads were pelleted as before and the supernatant removed. The elution step was repeated five more times and the supernatant fractions analysed by SDS-PAGE.

Animals

Female BALB/c mice were purchased from Harlan Olac UK Blackthorn, Bicester, UK, and used when at least 8 weeks of age.

Inoculations and viable counting or organ homogenates

Bacteria were grown in tryptic soy broth (Oxoid) supplemented with 100 µg/ml ampicillin as required. For intravenous inoculation, stationary cultures were diluted in PBS and animals were given approx. 10^6 cfu in a lateral tail vein in 0.2 ml. For oral inoculation, bacteria were grown in shaken overnight cultures, concentrated by centrifugation, and animals received approximately 5×10^9 cfu in 0.2 ml intragastrically via a gavage tube. The inoculum doses were checked by viable counts on tryptic soy agar.

For viable counts on organ homogenates, groups of 3 mice were sacrificed at intervals, the livers and spleen and (for orally inoculated mice) a pool of mesenteric lymph nodes were homogenised separately in 10 ml distilled water in a Colworth stomacher [C.E. Hormaeche Immunology 37, 311-318, 1979] and viable counts performed on tryptic soy agar supplemented with 100 µg/ml ampicillin.

Measurement of antibody responses

Antibodies were measured by solid phase immunoassay. 96-well-flat bottomed plates were coated with either 0.1 µg of TetC (a kind gift from Dr N Fairweather, the Wellcome Foundation, Beckenham UK) or 1 µg of recombinant P28 (a kind gift from Dr R Pierce, Pasteur Institute, Lille, France) in 100 µl of 0.1 M carbonate buffer, pH 9.6. After overnight incubation at 4°C the plates were incubated for 1 h at 37°C. Blocking of non-specific binding sites was carried out by incubation with 200 µl of 2% casein (BDH, Poole, UK) in PBS pH 7.0 for 1 h at 37°C. Plates were washed three times with 0.05% Tween 20 (Sigma) in PBS with a semiautomatic ELISA washer (Titertek, Flow/ICN, Herts UK). 100 µl of sera from inoculated mice diluted 1:20 in 2% casein was added to each well and the plates were incubated for one hour at 37°C. The plates were washed as above and 100 µl of horse radish peroxidase conjugated goat antimouse immunoglobulins (Dako, Bucks UK), diluted according to the manufacturer's instructions in 2% casein

washes were given with PBS alone. The plates were developed using 3,3',3,3'-tetramethylbenzidine dihydrochloride (Sigma) according to the manufacturer's instructions using phosphate/citrate buffer, pH 5.0 and 0.02% hydrogen peroxide. The plates were incubated for 10-15 min at 37°C after which the reaction was stopped with 25 µl 3M H₂SO₄ (BDH). The plates were read in an ELISA reader at 450 nm.

Measurement of T-cell responses

Spleens from mice vaccinated 6 months in advance were removed aseptically and single cell suspensions were prepared by mashing the spleens through a stainless steel sieve with the help of a plastic plunger. Cells were washed once in RPMI1640 medium (Flow/ICN) at 300xg and incubated in Gey's solution to lyse the red cells. White cells were washed twice more as above and resuspended in complete medium, i.e. RPMI1640 supplemented with 100 U/ml penicillin G (Flow/ICN), 100 µg/ml streptomycin (Flow/ICN), 2X10⁻⁵M B-mercapto-ethanol (Sigma), 1mM N-(2-hydroxyethyl-piperazine-N'-(2-ethanesulphonic acid) (HEPES) (Flow/ICN) and 10% heat inactivated newborn bovine serum (Northumbria Biolabs, Northumberland, UK). For isolation of T-cells, spleen cells were treated as above and after lysis of red cells the white cells were resuspended in warm (37°C) RPMI1640 and passed through a Wigzell glass bead column [H. Wigzell, et al Scand. J. Immunol 1: 75-87, 1972] .

Cells were plated at 2X10⁶/ml in a final volume of 200 µl of complete medium in 96-well plates in the presence of

the relevant antigens. These were either an alkali-treated whole cell soluble extract of S.typhimurium C5 prepared as described in Villarreal *et al.* [Microbial Pathogenesis 13: 305-315, 1992] at 20 µg/ml final concentration; TetC at 10 µg/ml; recombinant Schistosoma mansoni P28 at 50 µg/ml; and S. mansoni whole adult worm extract (a kind gift from Dr D Dunne, Cambridge University) at 20 µg/ml. Cells were incubated in a 95% humidity, 5% CO₂, 37°C atmosphere.

Feeder cells for T-cells for animals immunised with SL3261(pTECH1-P28) were obtained from syngeneic BALB/c naive spleens prepared as above. For mice immunised with pTETnir15, feeder cells were obtained from similarly immunised animals. After red cell lysis and two washes with RPMI1640 cells were X-ray irradiated at 2000 rads and washed twice more. These antigen presenting cells were resuspended in complete medium to give a final ratio of 1:1 with T-cells.

IL-2 production and assay

T-cell suspensions were plated as above. After two days, 50 µl of supernatant was harvested and added to 1×10^4 cells/well CTLL-2(IL-2 dependent) in 50 µl of medium. CTLL-2 cells were obtained from Dr J Ellis, University College, London UK and maintained in RPMI1640 supplemented as above, substituting the newborn bovine serum for foetal bovine serum. After 20 h, 20 µl of MTT at a concentration of 5 mg/ml in PBS were added. MTT transformation was measured as indicated elsewhere [Tada *et al.* J. Immunol. Methods 93: 157-165, 1986]. results were expressed as the

38

mean of the optical density of triplicates read at 570 nm using a reference filter of 630 nm. Significance was determined by Student's t-test.

BACTERIAL SAMPLE DEPOSITS

Salmonella typhimurium strains SL3261-pTECH1, SL3261-pTECH1-P28, SL3261-pTECH2, SL3261-pTECH2-P28 Octamer and PTE79 have been deposited at the National Collection of Type Cultures, 61 Colindale Avenue, London, NW9 5HT, UK, on 15th July 1993 under Deposit Numbers NCTC 12831, NCTC 12833, 12832, 12834 and 12837 respectively.

TABLE 1DNA SEQUENCES OF OLIGONUCLEOTIDES UTILISED IN THE
CONSTRUCTION OF THE TETC-HINGE VECTORSA). Primer 1. Sense PCR (21mer). (SEQ ID NO: 4)

SacII

5'AAA GAC TCC GCG GGC GAA GTT -3'

TETANUS TOXIN C FRAGMENT SEQ.

B).Primer 2. Anti-Sense PCR Primer (64mer). (SEQ ID NO: 5)

BamHI STOP SpeI XbaI HINGE REGION

5'- CTAT GGA TCC TTA ACT AGT GAT TCT AGA GGG CCC CGG CCC

GTC GTT GGT CCA ACC TTC ATC GGT -3'

TETANUS TOXIN C FRAGMENT SEQ. 3'-END

C). The pTECH2 Linker (SEQ ID NO: 6)

XbaI BamHI EcoRV HindIII SpeI Stop XBamHI*

5'-CTAGA GGATCC GATATC AAGCTT ACTAGT TAA T-3'

3'-T CCTAGG CTATAG TTCGAA TGATCA ATT ACTAG-5'

*This BamHI recognition sequence is now destroyed.

TABLE 2

T-Cell responses (IL-2/IL-4 production) elicited by alkali treated salmonella whole cell extract (C5NaOH), TetC, *Schistosoma mansoni* whole adult worm antigen (SWA), and recombinant P28 in mice immunised with SL3261(pTETnir15) or SL3261(pTECH1.P28).

Immunising strain	Stimulating antigen				
	none	C5NaOH	TetC	P28	SWA
SL3261 (pTETnir15)	2±4	67±5	41±1	0	0
SL3261 (pTECH1-P28)	6±2.6	109±10	50±8	25±8 p<0.001	17±6 p<0.01
Results expressed as (A ₅₇₀ -A ₆₃₀) x 1000±S.D.					

TABLE 3Oligonucleotide Sequences for HSV, FMDV, and SIV.HSV1 gD Gene

PCR Primer 1: 5'-AATGGATCCAAATATGCCCTGGCGGATGC-3'
(SEQ ID NO: 7)

PCR Primer 2: 5'-TTAACTAGTGTGTTTCGGGGTGGCCGGGGGAT-3'
(SEQ ID NO: 8)

FMDV VP1 Epitope

Oligo 1:
5'-GATCTAAATACTCTGCTTCTGGTTCTGGTGTTCGTGGTGAC
TTCGGTCTCTGGCTCCGCGTGTGCTCGTCAGCTGA-3'
(SEQ ID NO: 9)

Oligo 2:
5'-CTAGTCAGCTGACGAGCAACACGCGGAGCCAGAGAACCGAA
GTCACCACGAACACCAGAACCAGAAGCAGAGTATTTA-3
(SEQ ID NO: 10)

SIV gp120 Epitope

Oligo 1:
5'-GATCTAACATGACCGGTCTGAAACGTGATAAAACCAAAGAA
TACAACGAAACCTGGTACTCTACCA-3'
(SEQ ID NO: 11)

Oligo 2:
5'-CTAGTGGTAGAGTACCAGGTTTCGTTGTATTCTTTGGTTTT
ATCACGTTTCAGACCGGTCATGTTA-3'
(SEQ ID NO: 12)

Sm P28 Gene

PCR Primer 1: 5'-TAGTCTAGAATGGCTGGCGAGCATATCAAG-3'
(SEQ ID NO: 13)

PCR Primer 2: 5'-TTAGGATCCTTAGAAGGGAGTTGCAGGCCT-3'
(SEQ ID NO: 14)

Sm P28 Epitope

Oligo 1:
5'-GATCTAAACCGCAGGAAGAAAAAGAAAAAATCACCAAAGAAA
TCCTGAACGGCAAAA-3'
(SEQ ID NO: 15)

Oligo 2:
5'-CTAGTTTTGCCGTTTCAGGATTTCTTTGGTGATTTTTTCTTTTCT
TCCTGCGGTTTA-3'
(SEQ ID NO: 16)

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT:

- (A) NAME: MEDEVA HOLDINGS BV
- (B) STREET: CHURCHILL-LAAN 223
- (C) CITY: AMSTERDAM
- (E) COUNTRY: THE NETHERLANDS
- (F) POSTAL CODE (ZIP): 1078 ED

(ii) TITLE OF INVENTION: VACCINES

(iii) NUMBER OF SEQUENCES: 20

(iv) COMPUTER READABLE FORM:

- (A) MEDIUM TYPE: Floppy disk
- (B) COMPUTER: IBM PC compatible
- (C) OPERATING SYSTEM: PC-DOS/MS-DOS
- (D) SOFTWARE: PatentIn Release #1.0, Version #1.25 (EPO)

(vi) PRIOR APPLICATION DATA:

- (A) APPLICATION NUMBER: GB 9216317.9
- (B) FILING DATE: 31-JUL-1992

(vi) PRIOR APPLICATION DATA:

- (A) APPLICATION NUMBER: GB 9306398.0
- (B) FILING DATE: 26-MAR-1993

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 62

43

GTAGGGCC

68

(2) INFORMATION FOR SEQ ID NO: 2:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 68 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

AATTCAGGTA AATTTGATGT ACATCAAATG GTACCCCTTG CTGAATCGTT AAGGTAGGCG 60

GTAGGGCC 68

(2) INFORMATION FOR SEQ ID NO: 3:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 60 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

CTACCGCCTA CCTTAACGAT TCAGCAAGGG GTACCATTG ATGTACATCA AATTACCTG 60

(2) INFORMATION FOR SEQ ID NO: 4:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 21 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

44

(iii) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

AAAGACTCCG CGGGCGAAGT T

21

(2) INFORMATION FOR SEQ ID NO: 5:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 64 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

CTATGGATCC TTAAGTAGTG ATTCTAGAGG GCGGCGGCC GTCGTTGGTC CAACCTTCAT

60

CGGT

64

(2) INFORMATION FOR SEQ ID NO: 6:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 33 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: double

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:

CTAGAGGATC CGATATCAAG CTTACTAGTT AAT

33

(2) INFORMATION FOR SEQ ID NO: 7:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 29 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

45

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 7:

AATGGATCCA AATATGCCCT GCGGGATGC

29

(2) INFORMATION FOR SEQ ID NO: 8:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 31 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 8:

TAACTAGTGT TGTTGGGGT GCCCGGGGA T

31

(2) INFORMATION FOR SEQ ID NO: 9:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 78 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 9:

GATCTAAATA CTCTGCTTCT GGTCTGGTG TTCGTGGTGA CTTGGTTCT CTGGCTCCGC

60

GTGTTGCTCG TCAGCTGA

78

46

(2) INFORMATION FOR SEQ ID NO: 10:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 78 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 10:

CTAGTCAGCT GACGAGCAAC ACGCGGAGCC AGAGAACCGA AGTCACCACG AACACCAGAA	60
CCAGAAGCAG AGTATTTA	78

(2) INFORMATION FOR SEQ ID NO: 11:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 66 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 11:

GATCTAACAT GACCGGTCTG AAACGTGATA AAACCAAAGA ATACAACGAA ACCTGGTACT	60
CTACCA	66

(2) INFORMATION FOR SEQ ID NO: 12:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 66 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

47

(iii) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 12:

CTAGTGGTAG AGTACCAGGT TTCGTTGTAT TCTTTGGTTT TATCACGTTT CAGACCGGTC 60
ATGTTA 66

(2) INFORMATION FOR SEQ ID NO: 13:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 30 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 13:

TAGTCTAGAA TGGCTGGCGA GCATATCAAG 30

(2) INFORMATION FOR SEQ ID NO: 14:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 30 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 14:

TTAGGATCCT TAGAAGGGAG TTGCAGGCCT 30

(2) INFORMATION FOR SEQ ID NO: 15:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 57 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single

48

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 15:

GATCTAAACC GCAGGAAGAA AAAGAAAAAA TCACCAAAGA AATCCTGAAC GGCAAAA

57

(2) INFORMATION FOR SEQ ID NO: 16:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 57 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 16:

CTAGTTTTGC CGTTCAGGAT TTCTTTGGTG ATTTTCTT TTTCTTCCTG CGGTTTA

57

(2) INFORMATION FOR SEQ ID NO: 17:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 3754 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: double

(D) TOPOLOGY: circular

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 17:

TTCAGGTAAA TTTGATGTAC ATCAAATGGT ACCCCTTGCT GAATCGTTAA GGTAGGCGGT

60

AGGGCCCAGA TCTTAATCAT CCACAGGAGA CTTTCTGATG AAAAACCTTG ATTGTTGGGT

120

CGACAACGAA GAAGACATCG ATGTTATCCT GAAAAAGTCT ACCATTCTGA ACTTGGACAT	180
CAACAACGAT ATTATCTCCG ACATCTCTGG TTTCAACTCC TCTGTTATCA CATATCCAGA	240
TGCTCAATTG GTGCCGGGCA TCAACGGCAA AGCTATCCAC CTGGTTAACA ACGAATCTTC	300
TGAAGTTATC GTGCACAAGG CCATGGACAT CGAATACAAC GACATGTTCA ACAACTTCAC	360
CGTTAGCTTC TGGCTGCGCG TTCCGAAAGT TTCTGCTTCC CACCTGGAAC AGTACGGCAC	420
TAACGAGTAC TCCATCATCA GCTCTATGAA GAAACACTCC CTGTCCATCG GCTCTGGTTG	480
GTCTGTTTCC CTGAAGGGTA ACAACCTGAT CTGGACTCTG AAAGACTCCG CGGGCGAAGT	540
TCGTCAGATC ACTTTCCGCG ACCTGCCGGA CAAGTTCAAC GCGTACCTGG CTAACAAATG	600
GGTTTTTCATC ACTATCACTA ACGATCGTCT GTCTTCTGCT AACCTGTACA TCAACGGCGT	660
TCTGATGGGC TCCGCTGAAA TCACTGGTCT GGGCGCTATC CGTGAGGACA ACAACATCAC	720
TCTTAAGCTG GACCGTTGCA ACAACAACAA CCAGTACGTA TCCATCGACA AGTTCCGTAT	780
CTTCTGCAAA GCACTGAACC CGAAAGAGAT CGAAAACTG TATACCAGCT ACCTGTCTAT	840
CACCTTCCTG CGTGACTTCT GGGGTAACCC GCTGCGTTAC GACACCGAAT ATTACCTGAT	900
CCCGGTAGCT TCTAGCTCTA AAGACGTTCA GCTGAAAAAC ATCACTGACT ACATGTACCT	960
GACCAACGCG CCGTCCTACA CTAACGGTAA ACTGAACATC TACTACCGAC GTCTGTACAA	1020
CGGCCTGAAA TTCATCATCA AACGCTACAC TCCGAACAAC GAAATCGATT CTTTCGTTAA	1080
ATCTGGTGAC TTCATCAAAC TGTACGTTTC TTACAACAAC AACGAACACA TCGTTGGTTA	1140
CCCGAAAGAC GGTAACGCTT TCAACAACCT GGACAGAATT CTGCGTGTG GTTACAACGC	1200
TCCGGGTATC CCGCTGTACA AAAAAATGGA AGCTGTAAAA CTGCGTGACC TGAAAACTA	1260
CTCTGTTCAG CTGAACTGT ACGACGACAA AAACGCTTCT CTGGGTCTGG TTGGTACCCA	1320
CAACGGTCAG ATCGGTAACG ACCCGAACCG TGACATCCTG ATCGCTTCTA ACTGGTACTT	1380
CAACCACCTG AAAGACAAAA TCCTGGGTTG CGACTGGTAC TTCGTTCCGA CCGATGAAGG	1440
TTGGACCAAC GACGGGCCGG GGCCCTCTAG AATCACTAGT TAAGGATCCG CTAGCCCGCC	1500
TAATGAGCGG GCTTTTTTTT CTCGGGCAGC GTTGGGTCTT GGCCACGGGT GCGCATGATC	1560
GTGCTCCTGT CGTTGAGGAC CCGGCTAGGC TGGCGGGGTT GCCTTACTGG TTAGCAGAAT	1620
GAATCACCGA TACGCGAGCG AACGTGAAGC GACTGCTGCT GCAAAACGTC TGCGACCTGA	1680
GCAACAACAT GAATGGTCTT CGGTTTCCGT GTTTCGTAAA GTCTGGAAAC GCGGAAGTCA	1740
GCGCTCTTCC GCTTCCTCGC TCACTGACTC GCTGCGCTCG GTCGTTCCGC TGCGGCGAGC	1800

GGTATCAGCT CACTCAAAGG CGGTAATACG GTTATCCACA GAATCAGGGG ATAACGCAGG	1860
AAAGAACATG TGAGCAAAAG GCCAGCAAAA GGCCAGGAAC CGTAAAAAGG CCGCGTTGCT	1920
GGCGTTTTTC CATAGGCTCC GCCCCCTGA CGAGCATCAC AAAAATCGAC GCTCAAGTCA	1980
GAGGTGGCGA AACCCGACAG GACTATAAAG ATACCAGGCG TTTCCCCCTG GAAGCTCCCT	2040
CGTGCGCTCT CCTGTTCCGA CCCTGCCGCT TACCGGATAC CTGTCCGCCT TTCTCCCTTC	2100
GGAAGCGTG GCGCTTTCTC AATGCTCAG CTGTAGGTAT CTCAGTTCGG TGTAGGTCGT	2160
TCGCTCCAAG CTGGGCTGTG TGCACGAACC CCCCCTCAG CCGACCGCT GCGCCTTATC	2220
CGGTAAGTAT CGTCTTGAGT CCAACCCGGT AAGACACGAC TTATCGCCAC TGGCAGCAGC	2280
CACTGGTAAC AGGATTAGCA GAGCGAGGTA TGTAGGCGGT GCTACAGAGT TCTTGAAGTG	2340
GTGGCCTAAC TACGGCTACA CTAGAAGGAC AGTATTTGGT ATCTGCGCTC TGCTGAAGCC	2400
AGTTACCTTC GGAAAAAGAG TTGGTAGCTC TTGATCCGGC AAACAAACCA CCGCTGGTAG	2460
CGGTGGTTTT TTTGTTTGCA AGCAGCAGAT TACGCGCAGA AAAAAAGGAT CTCAAGAAGA	2520
TCCTTTGATC TTTTCTACGG GGTCTGACGC TCAGTGGAAC GAAAACTCAC GTTAAGGGAT	2580
TTTGGTCATG AGATTATCAA AAAGGATCTT CACCTAGATC CTTTTAAATT AAAAATGAAG	2640
TTTTAAATCA ATCTAAAGTA TATATGAGTA AACTTGGTCT GACAGTTACC AATGCTTAAT	2700
CAGTGAGGCA CCTATCTCAG CGATCTGTCT ATTTCTGTTCA TCCATAGTTG CCTGACTCCC	2760
CGTCGTGTAG ATAACACGA TACGGGAGGG CTTACCATCT GGCCCCAGTG CTGCAATGAT	2820
ACCGCGAGAC CCACGCTCAC CGGCTCCAGA TTTATCAGCA ATAAACCAGC CAGCCGAAG	2880
GGCCGAGCGC AGAAGTGGTC CTGCAACTTT ATCCGCCTCC ATCCAGTCTA TTAATTGTTG	2940
CCGGAAGCT AGAGTAAGTA GTTCGCCAGT TAATAGTTTG CGCAACGTTG TTGCCATTGC	3000
TGCAGGCATC GTGGTGTAC GTCGTCGTT TGGTATGGCT TCATTCAGCT CCGGTTCCCA	3060
ACGATCAAGG CGAGTTACAT GATCCCCAT GTTGTGCAAA AAAGCGGTTA GCTCCTTCGG	3120
TCCTCCGATC GTTGTAGAA GTAAGTTGGC CGCAGTGTTA TCACTCATGG TTATGGCAGC	3180
ACTGCATAAT TCTCTTACTG TCATGCCATC CGTAAGATGC TTTTCTGTGA CTGGTGAGTA	3240
CTCAACCAAG TCATTCTGAG AATAGTGTAT GCGGCGACCG AGTTGCTCTT GCCCGGCGTC	3300
AACACGGGAT AATACCGCGC CACATAGCAG AACTTTAAAA GTGCTCATCA TTGGAACG	3360
TTCTTCGGGG CGAAACTCT CAAGGATCTT ACCGCTGTTG AGATCCAGTT CGATGTAACC	3420
CACTCGTGCA CCAACTGAT CTTGAGCATC TTTTACTTTC ACCAGCGTTT CTGGGTGAGC	3480

AAAAACAGGA AGGCAAAATG CCGCAAAAAA GGGAATAAGG GCGACACGGA AATGTTGAAT	3540
ACTCATACTC TTCCTTTTTC AATATTATTG AAGCATTTAT CAGGGTTATT GTCTCATGAG	3600
CGGATACATA TTTGAATGTA TTTAGAAAAA TAAACAAATA GGGGTTCCGC GCACATTTCC	3660
CCGAAAAGTG CCACCTGACG TCTAAGAAAC CATTATTATC ATGACATTAA CCTATAAAAA	3720
TAGGCGTATC ACGAGGCCCT TCGTCTTCA AGAA	3754

(2) INFORMATION FOR SEQ ID NO: 18:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 3769 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: double
- (D) TOPOLOGY: circular

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 18:

TTCAGGTAAA TTTGATGTAC ATCAAATGGT ACCCCTTGCT GAATCGTTAA GGTAGGCGGT	60
AGGGCCCAGA TCTTAATCAT CCACAGGAGA CTTTCTGATG AAAACCTTG ATTGTTGGGT	120
CGACAACGAA GAAGACATCG ATGTTATCCT GAAAAAGTCT ACCATTCTGA ACTTGGACAT	180
CAACAACGAT ATTATCTCCG ACATCTCTGG TTTCAACTCC TCTGTTATCA CATATCCAGA	240
TGCTCAATTG GTGCCGGGCA TCAACGGCAA AGCTATCCAC CTGGTTAACA ACGAATCTTC	300
TGAAGTTATC GTGCACAAGG CCATGGACAT CGAATACAAC GACATGTTCA ACAACTTCAC	360
CGTTAGCTTC TGGCTGCGCG TTCCGAAAGT TTCTGCTTCC CACCTGGAAC AGTACGGCAC	420
TAACGAGTAC TCCATCATCA GCTCTATGAA GAAACACTCC CTGTCCATCG GCTCTGGTTG	480
GTCTGTTTCC CTGAAGGGTA ACAACCTGAT CTGGACTCTG AAAGACTCCG CGGGCGAAGT	540
TCGTCAGATC ACTTCCGCG ACCTGCCGGA CAAGTTCAAC GCGTACCTGG CTAACAAATG	600
GGTTTTCATC ACTATCACTA ACGATCGTCT GTCTTCTGCT AACCTGTACA TCAACGGCGT	660
TCTGATGGGC TCCGCTGAAA TCACTGGTCT GGGCGCTATC CGTGAGGACA ACAACATCAC	720
TCTTAAGCTG GACCGTTGCA ACAACAACAA CCACTACGTA TCCATCGACA AGTTCGGTAT	780
CTTCTGCAAA GCACTGAACC CGAAAGAGAT CGAAAACTG TATACCAGCT ACCTGTCTAT	840

CACCTTCCTG CGTGACTTCT GGGGTAACCC GCTGCGTTAC GACACCGAAT ATTACCTGAT	900
CCCGGTAGCT TCTAGCTCTA AAGACGTTCA GCTGAAAAAC ATCACTGACT ACATGTACCT	960
GACCAACGCG CCGTCCTACA CTAACGGTAA ACTGAACATC TACTACCGAC GTCTGTACAA	1020
CGGCCTGAAA TTCATCATCA AACGCTACAC TCCGAACAAC GAAATCGATT CTTTCGTTAA	1080
ATCTGGTGAC TTCATCAAAC TGTACGTTTC TTACAACAAC AACGAACACA TCGTTGGTTA	1140
CCCgAAAGAC GGTAACGCTT TCAACAACCT GGACAGAATT CTGCGTGTTG GTTACAACGC	1200
TCCGGGTATC CCGCTGTACA AAAAAATGGA AGCTGTTAAA CTGCGTGACC TGAAAACTA	1260
CTCTGTTTCA CTGAACTGT ACGACGACAA AAACGCTTCT CTGGGTCTGG TTGGTACCCA	1320
CAACGGTCAG ATCGGTAACG ACCCGAACCG TGACATCCTG ATCGCTTCTA ACTGGTACTT	1380
CAACCACCTG AAAGACAAAA TCCTGGGTTG CGACTGGTAC TTCGTTCCGA CCGATGAAGG	1440
TTGGACCAAC GACGGGCCGG GGCCTCTAG AGGATCCGAT ATCAAGCTTA CTAGTTAATG	1500
ATCCGCTAGC CCGCCTAATG AGCGGGCTTT TTTTCTCGG GCAGCGTTGG GTCCTGGCCA	1560
CGGGTGCGCA TGATCGTGCT CCTGTCGTTG AGGACCCGGC TAGGCTGGCG GGGTTGCCTT	1620
ACTGGTTAGC AGAATGAATC ACCGATACGC GAGCGAACGT GAAGCGACTG CTGCTGCAAA	1680
ACGTCTGCGA CCTGAGCAAC AACATGAATG GTCTTCGGTT TCCGTGTTTC GTAAAGTCTG	1740
GAAACGCGGA AGTCAGCGCT CTTCCGCTTC CTCGCTCACT GACTCGCTGC GCTCGGTCGT	1800
TCGGCTGCGG CGAGCGGTAT CAGCTCACTC AAAGGCGGTA ATACGGTTAT CCACAGAATC	1860
AGGGGATAAC GCAGGAAAGA ACATGTGAGC AAAAGGCCAG CAAAAGGCCA GGAACCGTAA	1920
AAAGGCCGCG TTGCTGGCGT TTTTCCATAG GCTCCGCCCC CCTGACGAGC ATCACAAAAA	1980
TCGACGCTCA AGTCAGAGGT GCGGAAACCC GACAGGACTA TAAAGATACC AGGCGTTTCC	2040
CCCTGGAAGC TCCCTCGTGC GCTCTCCTGT TCCGACCCTG CCGCTTACCG GATACCTGTC	2100
CGCCTTTCTC CCTTCGGGAA GCGTGGCGCT TTCTCAATGC TCACGCTGTA GGTATCTCAG	2160
TTCGGTGTAG GTCGTTGCT CCAAGCTGGG CTGTGTGCAC GAACCCCCCG TTCAGCCCGA	2220
CCGCTGCGCC TTATCCGGTA ACTATCGTCT TGAGTCCAAC CCGGTAAGAC ACGACTTATC	2280
GCCACTGGCA GCAGCCACTG GTAACAGGAT TAGCAGAGCG AGGTATGTAG GCGGTGCTAC	2340
AGAGTTCTTG AAGTGGTGGC CTAACACGG CTACACTAGA AGGACAGTAT TTGGTATCTG	2400
CGCTCTGCTG AAGCCAGTTA CCTTCGGAAG AAGAGTTGGT AGCTCTTGAT CCGGCAACA	2460
AACCACCGCT GGTAGCGGTG GTTTTTTTGT TTGCAAGCAG CAGATTACGC GCAGAAAAAA	2520

AGGATCTCAA GAAGATCCTT TGATCTTTTC TACGGGGTCT GACGCTCAGT GGAACGAAAA	2580
CTCACGTTAA GGGATTTTGG TCATGAGATT ATCAAAAAGG ATCTTCACCT AGATCCTTTT	2640
AAATTAAAAA TGAAGTTTTA AATCAATCTA AAGTATATAT GAGTAAACTT GGTCTGACAG	2700
TTACCAATGC TTAATCAGTG AGGCACCTAT CTCAGCGATC TGTCTATTTT GTTCATCCAT	2760
AGTTGCCTGA CTCCCCGTCG TG TAGATAAC TACGATACGG GAGGGCTTAC CATCTGGCCC	2820
CAGTGCTGCA ATGATACCGC GAGACCCACG CTCACCGGCT CCAGATTTAT CAGCAATAAA	2880
CCAGCCAGCC GGAAGGGCCG AGCGCAGAAG TGGTCTGCA ACTTTATCCG CCTCCATCCA	2940
GTCTATTAAT TGTTGCCGGG AAGCTAGAGT AAGTAGTTTC CCAGTTAATA GTTTGCGCAA	3000
CGTTGTTGCC ATTGCTGCAG GCATCGTGGT GTCACGCTCG TCGTTTGGTA TGGCTTCATT	3060
CAGCTCCGGT TCCCAACGAT CAAGGCGAGT TACATGATCC CCCATGTTGT GCAAAAAAGC	3120
GGTTAGCTCC TTCGGTCCTC CGATCGTTGT CAGAAGTAAG TTGGCCGCAG TGTTATCACT	3180
CATGGTTATG GCAGCACTGC ATAATTCTCT TACTGTCATG CCATCCGTAA GATGCTTTTC	3240
TGTGACTGGT GAGTACTCAA CCAAGTCATT CTGAGAATAG TGTATGCGGC GACCGAGTTG	3300
CTCTTGCCCG GCGTCAACAC GGGATAATAC CGCGCCACAT AGCAGAACTT TAAAAGTGCT	3360
CATCATTGGA AAACGTTCTT CGGGGCGAAA ACTCTCAAGG ATCTTACCGC TGTTGAGATC	3420
CAGTTCGATG TAACCCACTC GTGCACCCAA CTGATCTTCA GCATCTTTTA CTTTCACCAG	3480
CGTTTCTGGG TGAGCAAAAA CAGGAAGGCA AAATGCCGCA AAAAAGGGAA TAAGGGCGAC	3540
ACGGAAATGT TGAATACTCA TACTCTTCCT TTTTCAATAT TATTGAAGCA TTTATCAGGG	3600
TTATTGTCTC ATGAGCGGAT ACATATTTGA ATGTATTTAG AAAAATAAAC AAATAGGGGT	3660
TCCGCGCACA TTTCCCCGAA AAGTGCCACC TGACGTCTAA GAAACCATTA TTATCATGAC	3720
ATTAACCTAT AAAAATAGGC GTATCACGAG GCCCTTTCGT CTTCAAGAA	3769

(2) INFORMATION FOR SEQ ID NO: 19:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 38 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: double
- (D) TOPOLOGY: circular

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: NO

54

(v) FRAGMENT TYPE: internal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 19:

TCTAGAGGAT CCGATATCAA GCTTACTAGT TAATGATC

38

(2) INFORMATION FOR SEQ ID NO: 20:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 14 amino acids

(B) TYPE: amino acid

(D) TOPOLOGY: circular

(ii) MOLECULE TYPE: peptide

(v) FRAGMENT TYPE: internal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 20:

Gly Pro Gly Pro Ser Arg Gly Ser Asp Ile Lys Leu Thr Ser
1 5 10

CLAIMS

1. A DNA construct comprising a promoter sequence operably linked to a DNA sequence encoding first and second proteins linked by a hinge region, characterised in that the promoter sequence is one having activity which is induced in response to a change in the surrounding environment.
2. A DNA construct according to claim 1 wherein the proteins are polypeptide immunogens.
3. A DNA construct according to claim 1 wherein the first heterologous protein is an antigenic sequence comprising tetanus toxin fragment C or epitopes thereof.
4. A DNA construct comprising a promoter sequence operably linked to a DNA sequence encoding linked first and second heterologous proteins wherein the first heterologous protein is an antigenic sequence comprising tetanus toxin fragment C or one or more epitopes thereof.
5. A DNA construct according to Claim 4 wherein the promoter sequence is one having activity which is induced in response to a change in surrounding environment.
6. A DNA construct according to any one of the preceding Claims wherein the first and second proteins are heterologous.

7. A DNA construct according to Claim 4 or Claim 5 where the first and second proteins are linked by a hinge region which is a discrete region heterologous to each of said proteins.
8. A DNA construct according to any one of the preceding Claims wherein the promoter sequence has activity which is induced by anaerobic conditions.
9. A DNA construct according to claim 7 wherein the promoter sequence is the nirB promoter or a part or derivative thereof which is capable of promoting expression of a coding sequence under anaerobic conditions.
10. A DNA construct according to any one of the preceding claims wherein the second protein is an antigenic sequence which is derived from a virus, bacterium, fungus, yeast or parasite.
11. A DNA construct according to Claim 7 wherein the antigenic sequence comprises the P28 antigen of Shistosoma mansoni or an epitope thereof, or an antigenic sequence derived from human papilloma virus, Herpes simplex virus, foot and mouth disease virus or simian immuno-deficiency virus.
12. A DNA construct comprising a promoter sequence whose activity is induced in response to a change in the surrounding environment, said promoter sequence being operably linked to a DNA sequence encoding a first antigenic sequence and a hinge region, and at or adjacent the 3'-end thereof one or more

restriction sites for the introduction of a second antigenic sequence.

13. A DNA construct comprising a promoter sequence operably linked to a first DNA sequence encoding Tetanus toxin C fragment, or one or more epitopes thereof, and a hinge region as defined in Claim 12.
14. A DNA construct according to Claim 13 wherein the promoter is one having activity which is induced in response to a change in the surrounding environment.
15. A DNA construct according to any one of Claims 12 to 14 wherein the promoter sequence has activity which is induced by anaerobic conditions.
16. A DNA construct according to claim 15 wherein the promoter is the nirB promoter or a part or derivative thereof which is capable of promoting expression of a sequence under anaerobic condition.
17. A replicable expression vector, for example suitable for use in bacteria, containing a DNA construct as defined in any one of Claims 1 to 16.
18. A bacterium transformed with an expression vector as defined in claim 17.

19. A bacterium according to claim 18 which is attenuated.
20. A process for the preparation of an attenuated bacterium according to Claim 19 which comprises transforming an attenuated bacterium with a DNA construct as defined in any one of claims 1 to 16.
21. A fusion protein comprising first and second proteins linked by a hinge region, the fusion protein being expressable by a replicable expression vector as defined in Claim 17.
22. A fusion protein comprising Tetanus toxin fragment C or one or more epitopes thereof linked to a second heterologous protein.
23. A fusion protein according to Claim 22 in substantially pure form.
24. A vaccine composition comprising an attenuated bacterium as defined in Claim 19, or a fusion protein as defined in Claim 22 or Claim 23, and a pharmaceutically acceptable carrier.

1/23

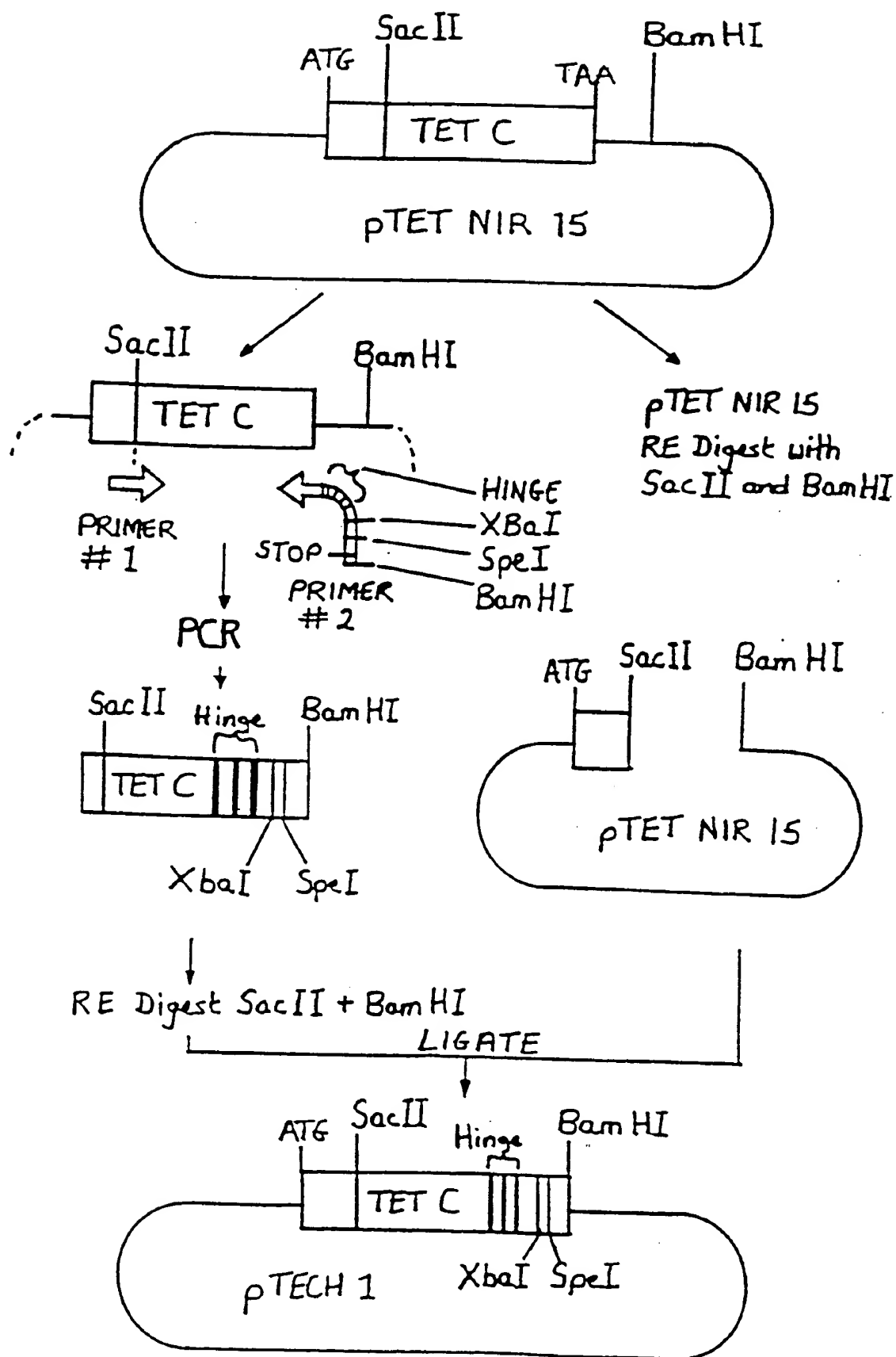


FIGURE 1

2/23

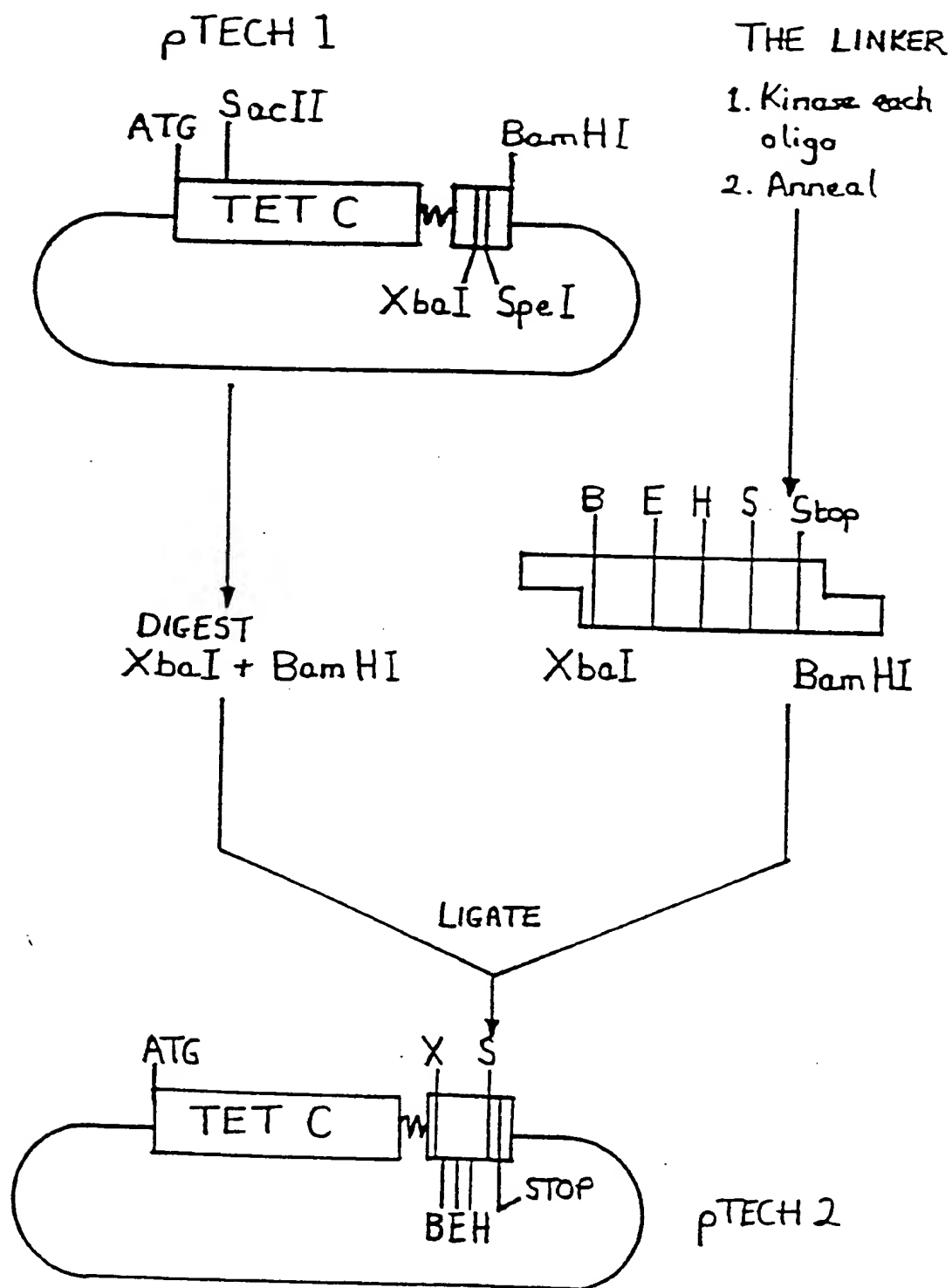


FIGURE 2

SUBSTITUTE SHEET

3/23

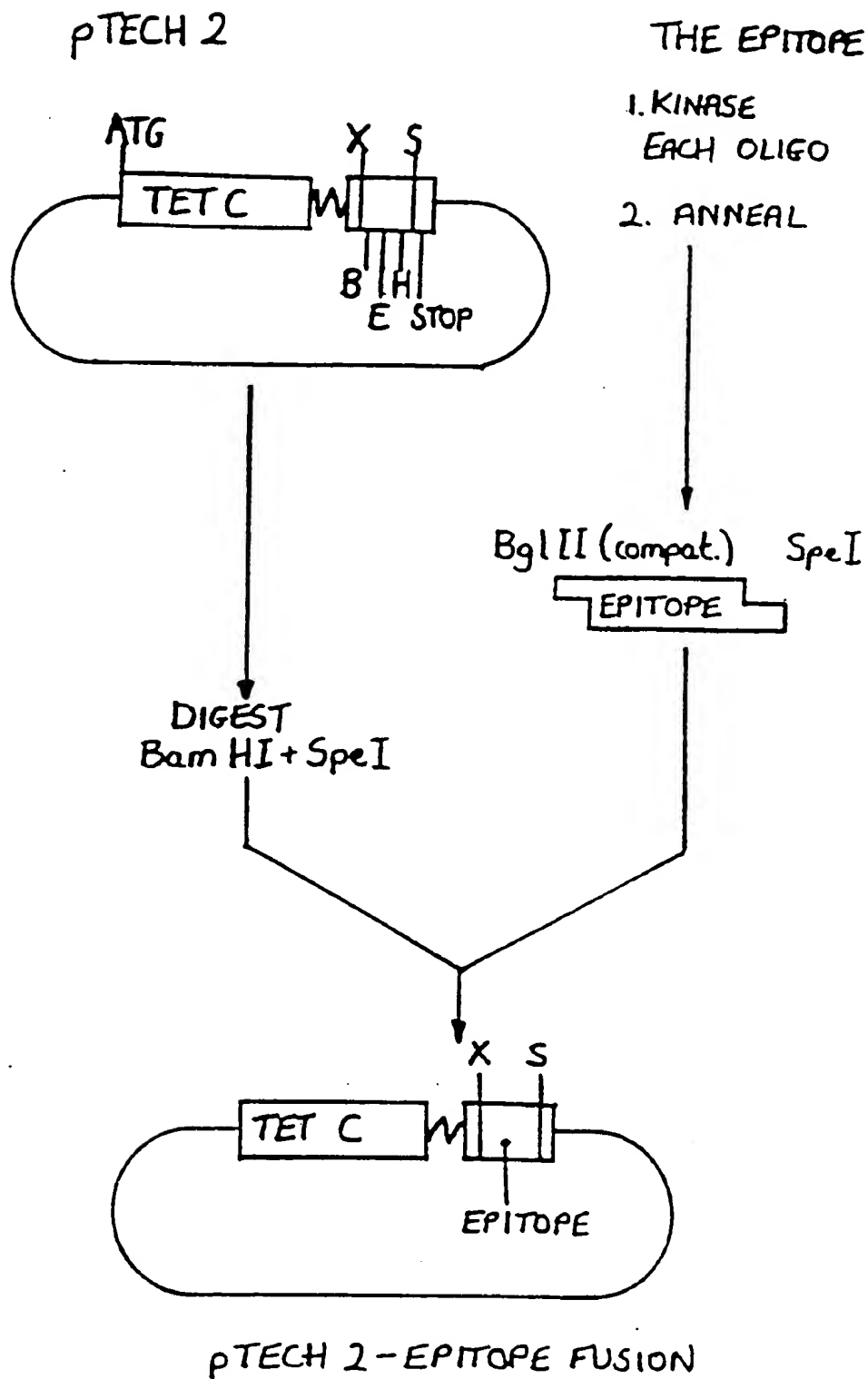


FIGURE 3

4/23

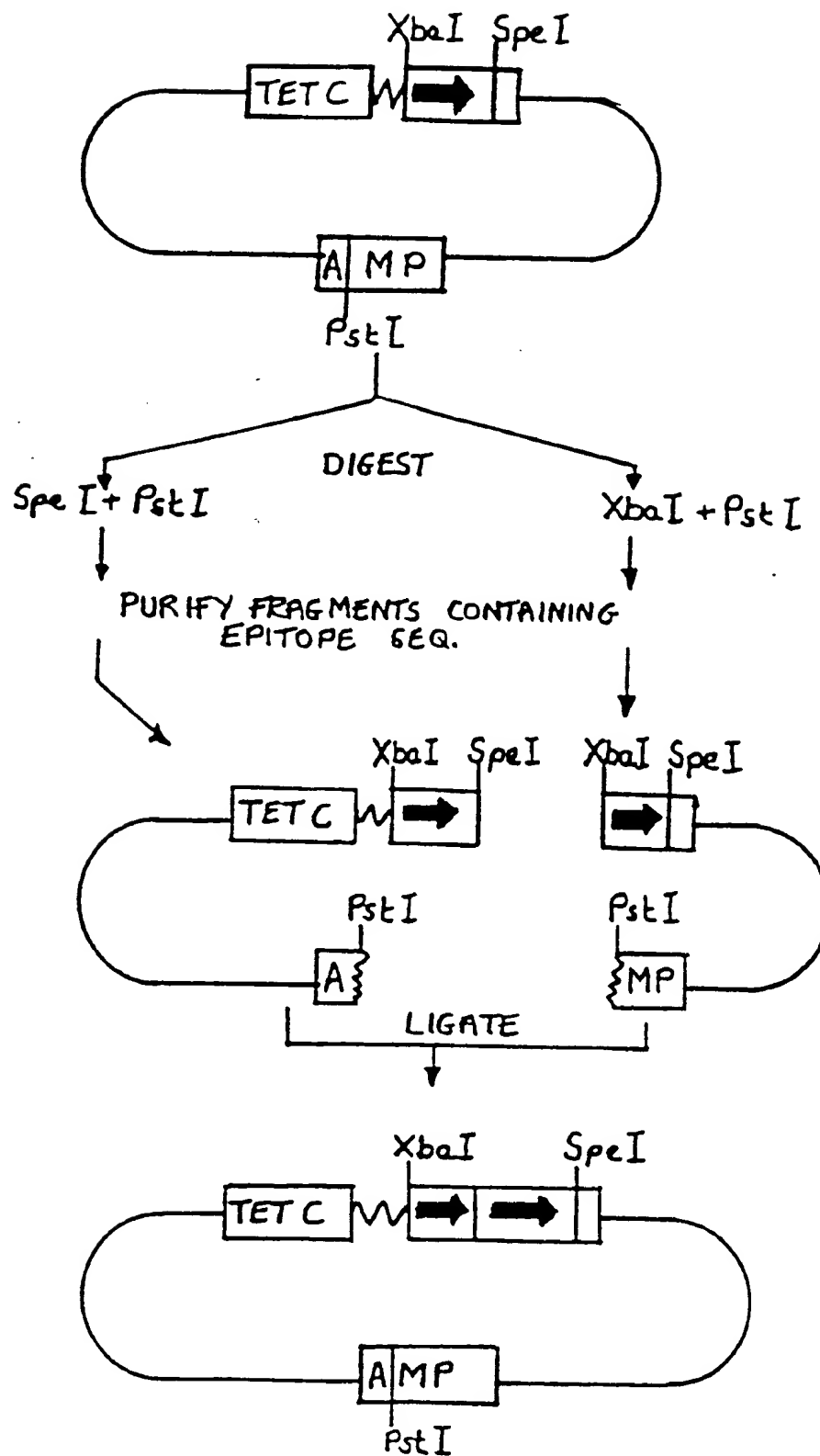


FIGURE 4

5/23

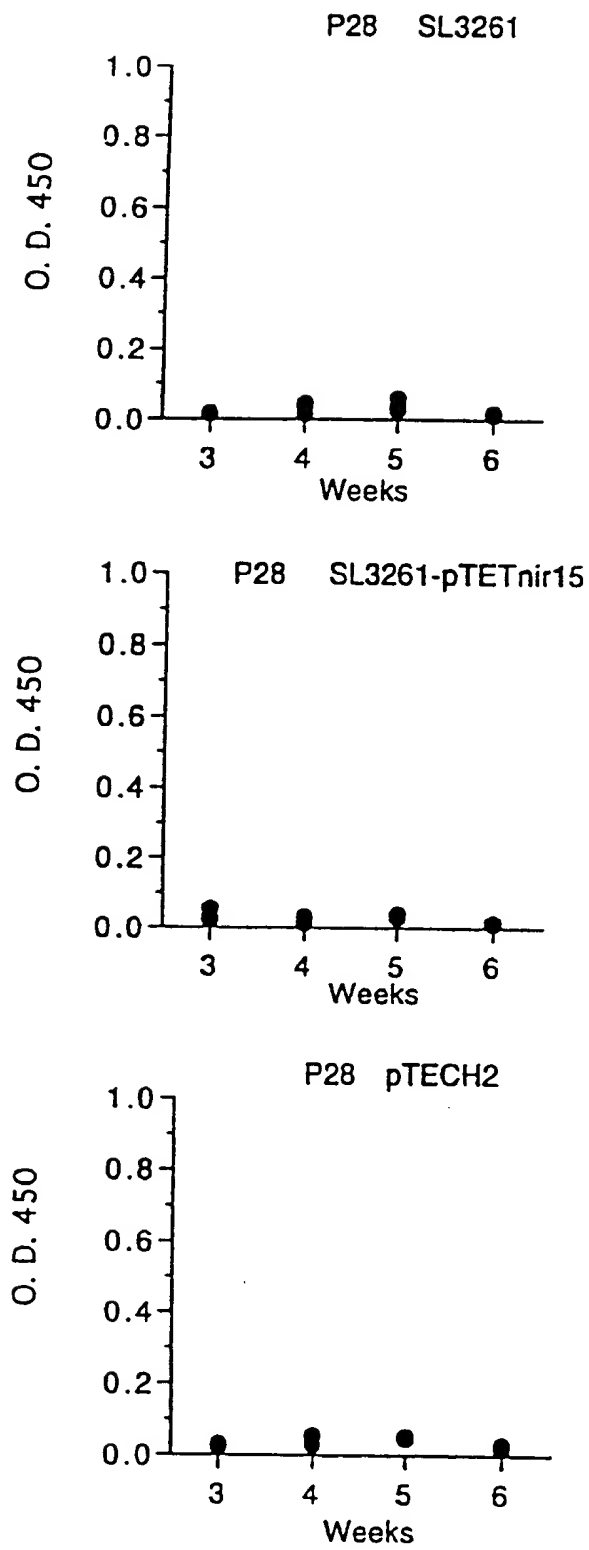


Figure 5

SUBSTITUTE SHEET

6/23

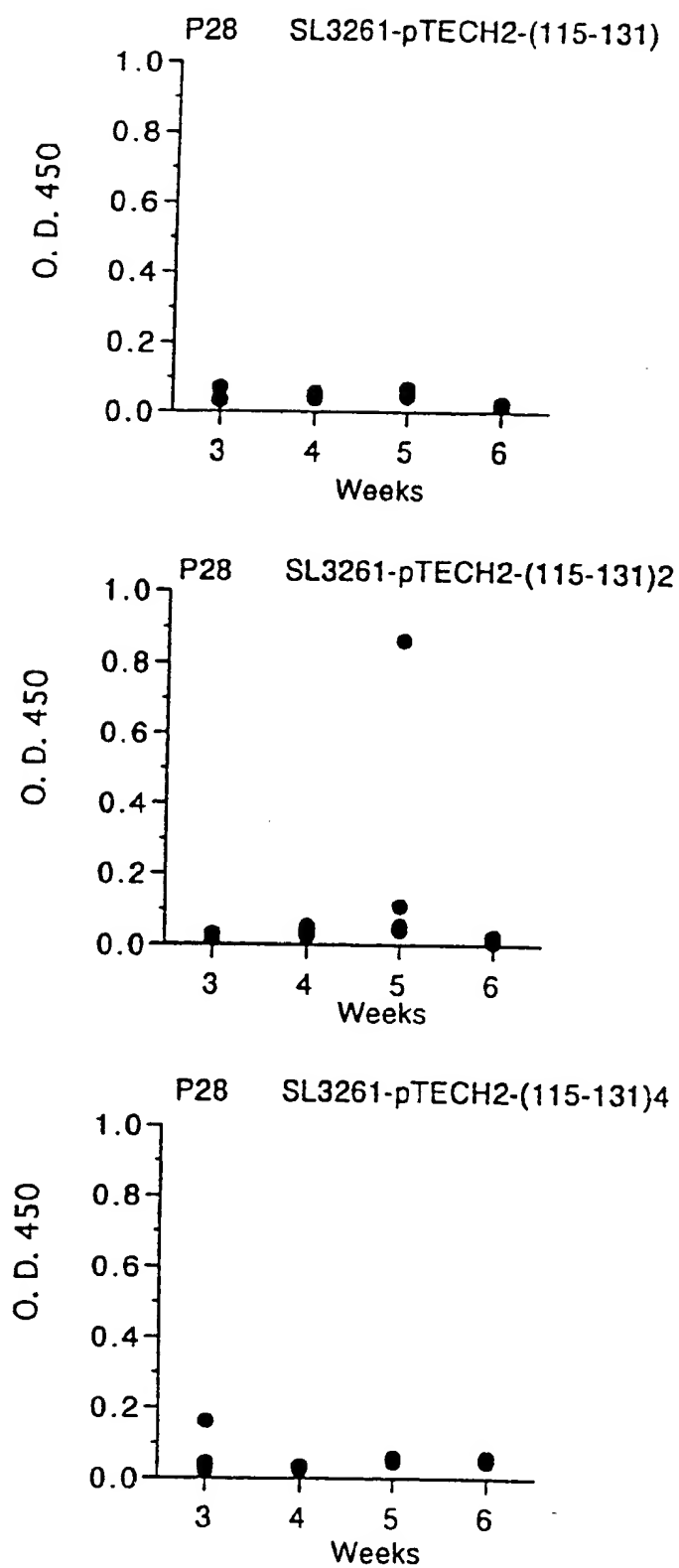


Figure 5 continued

SUBSTITUTE SHEET

7/23

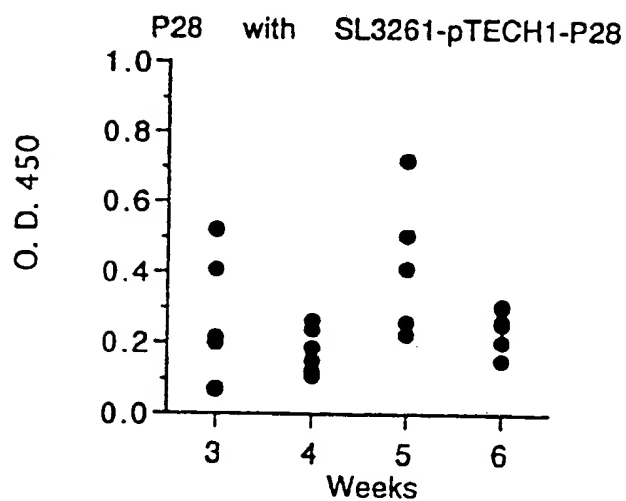
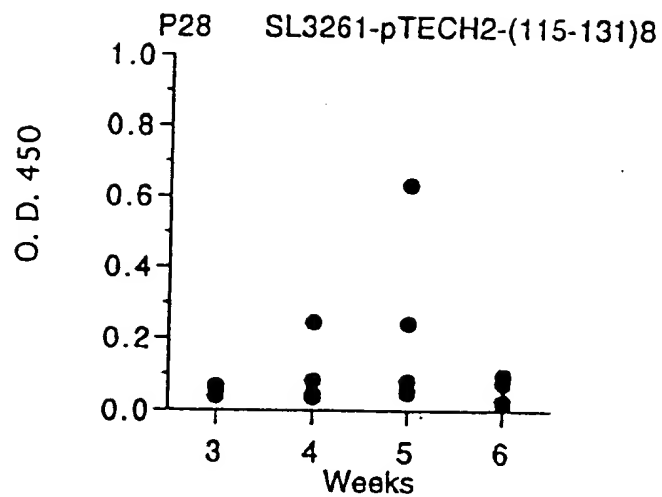


Figure 5 continued

SUBSTITUTE SHEET

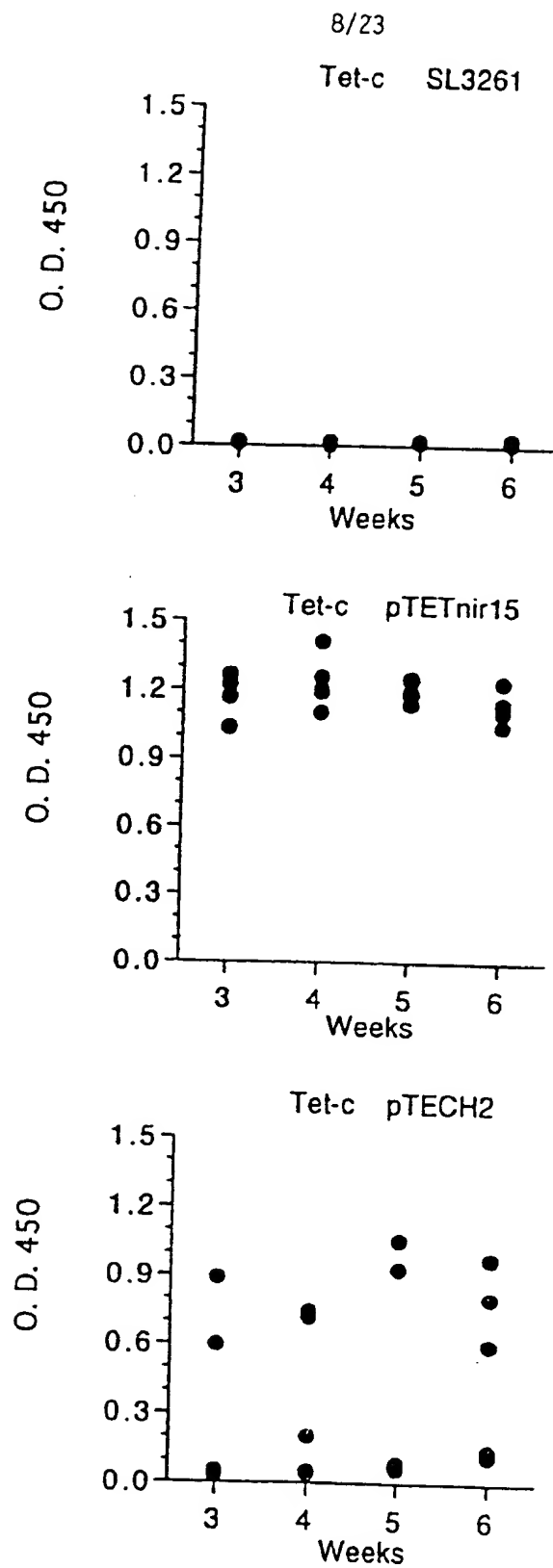


Figure 6

SUBSTITUTE SHEET

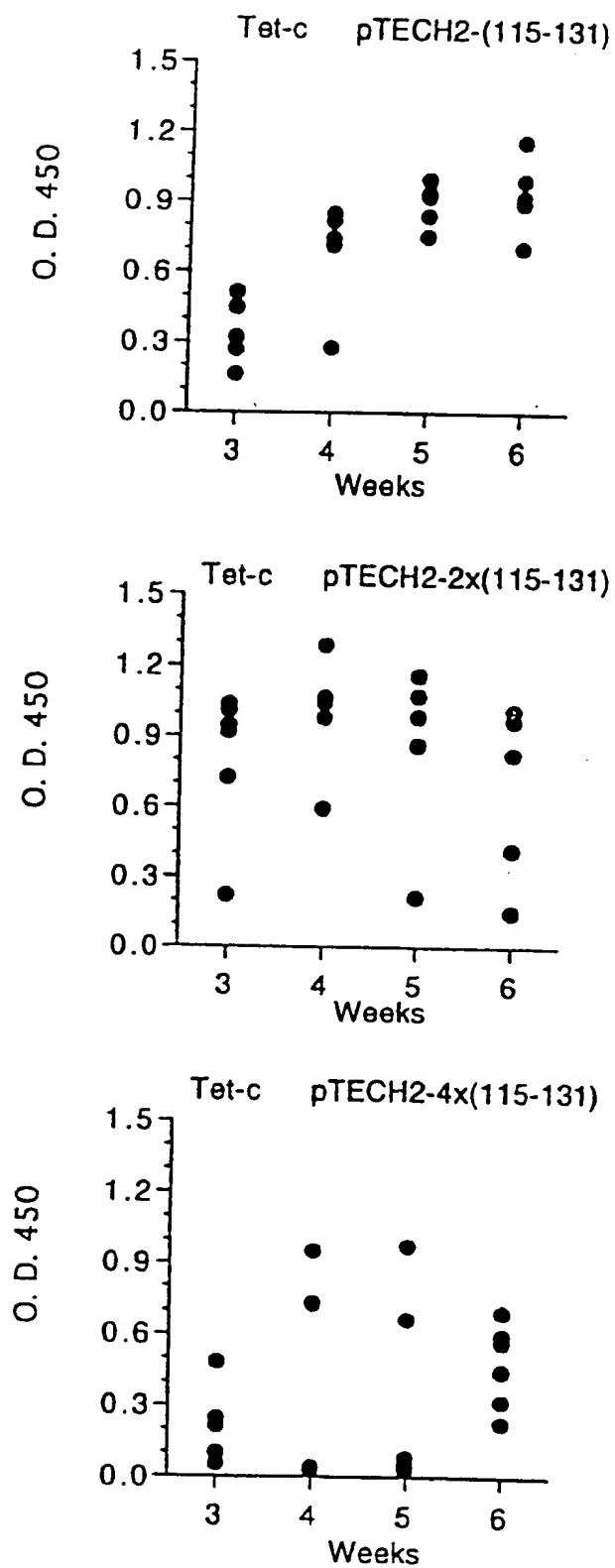


Figure 6 continued

10/23

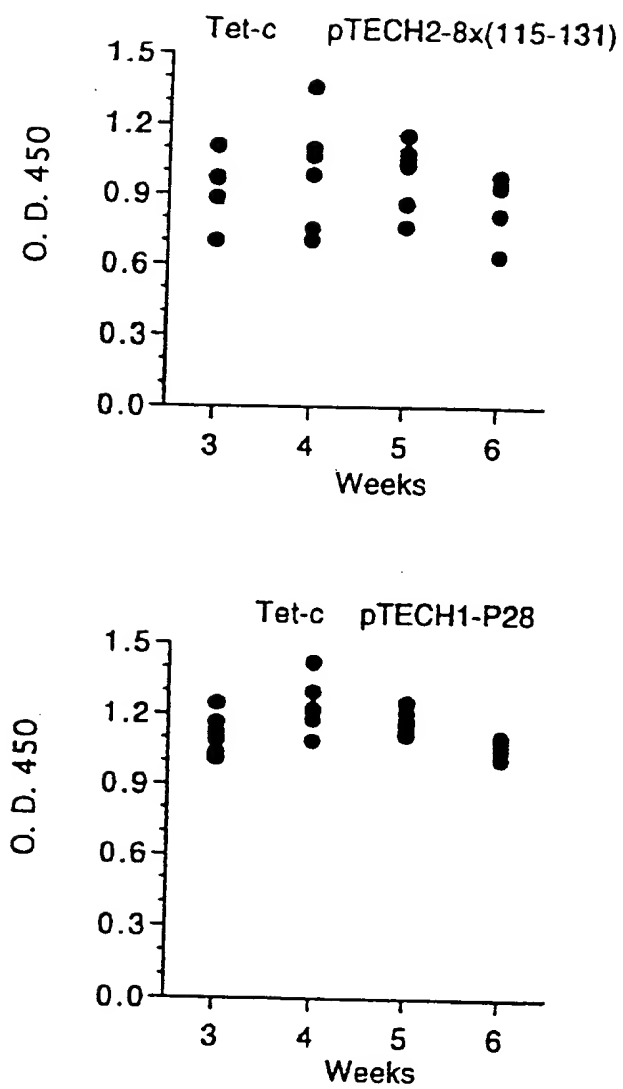


Figure 6 continued

SUBSTITUTE SHEET

11/23

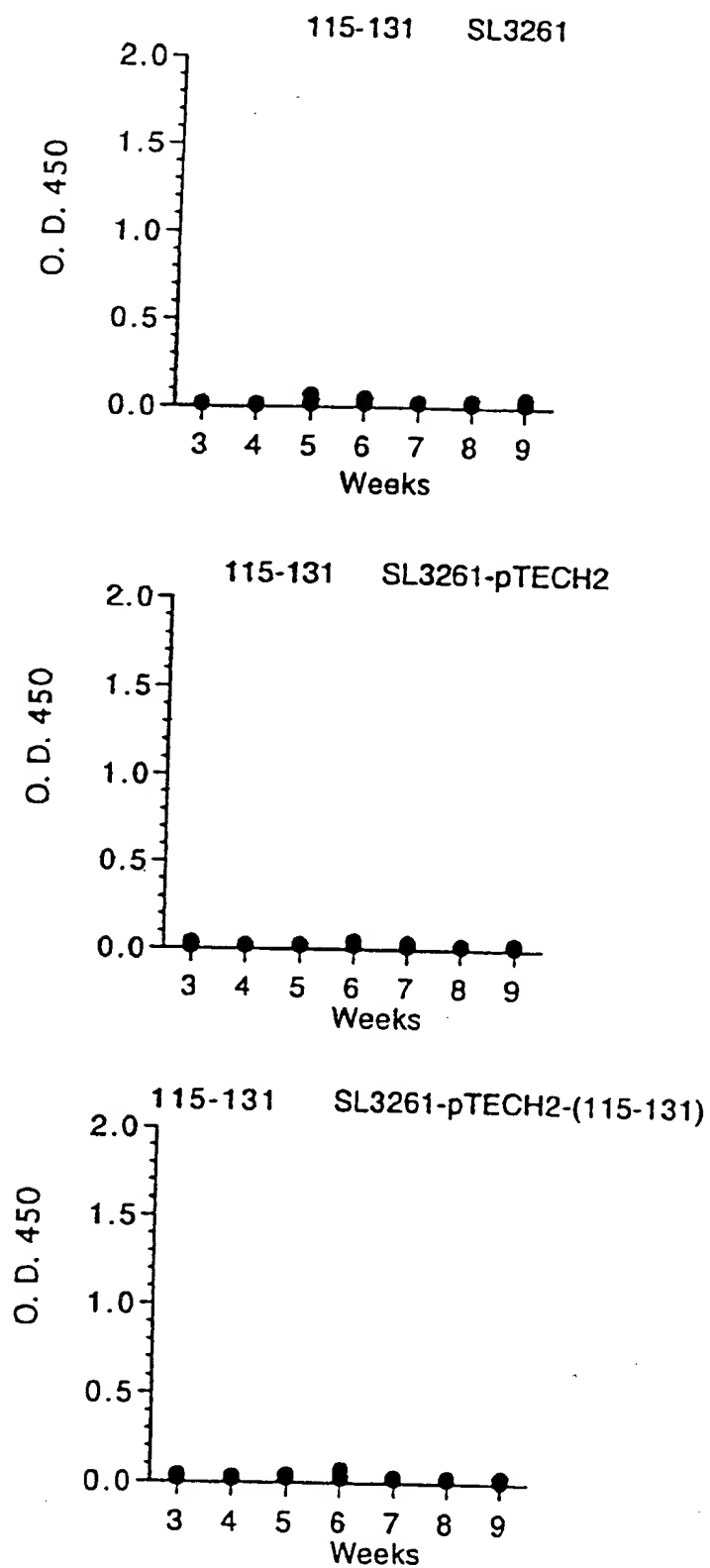


Figure 7

SUBSTITUTE SHEET

12/23

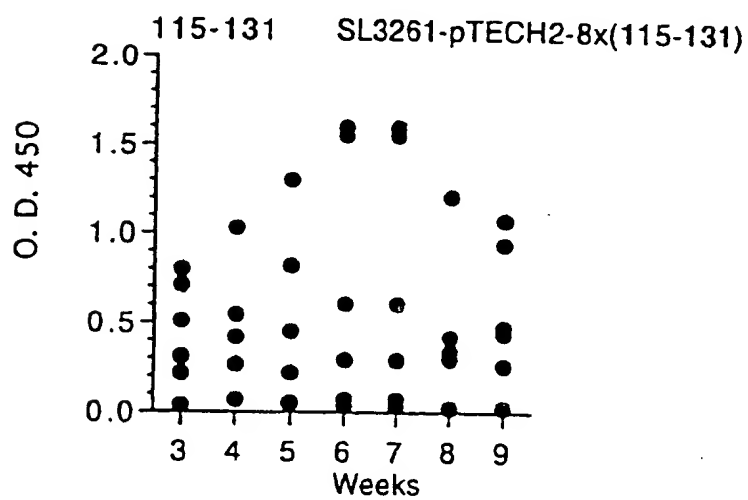
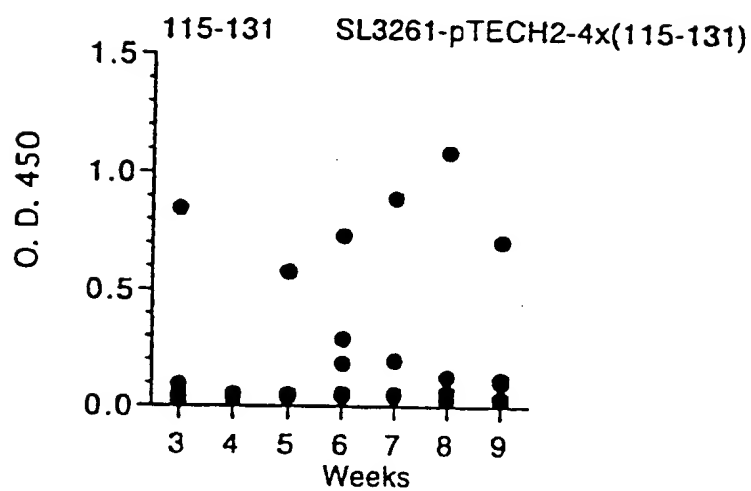
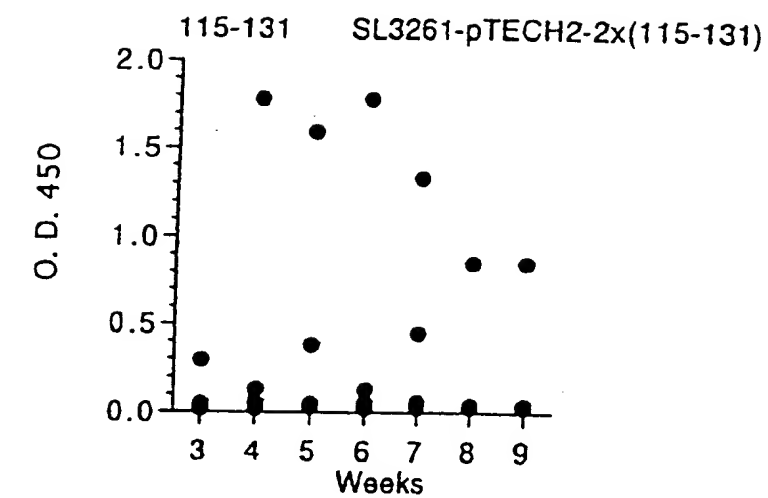


Figure 7 continued

SUBSTITUTE SHEET

13/23

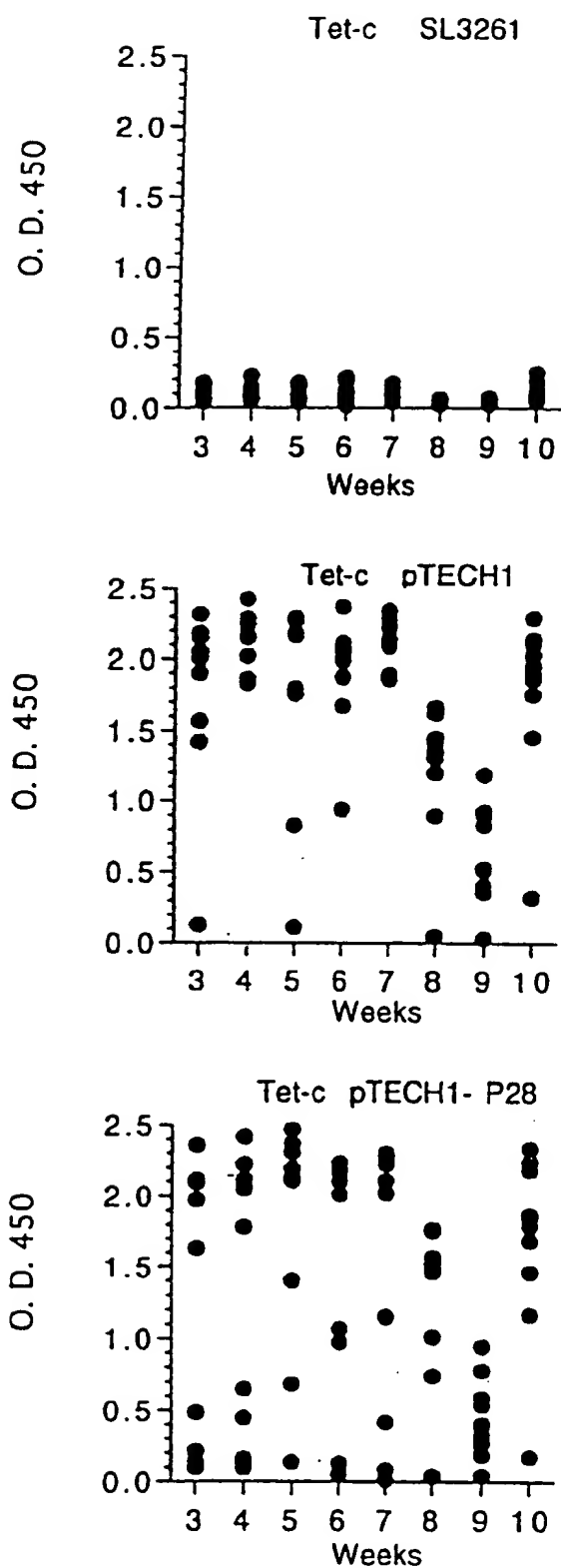


Figure 8

SUBSTITUTE SHEET

14/23

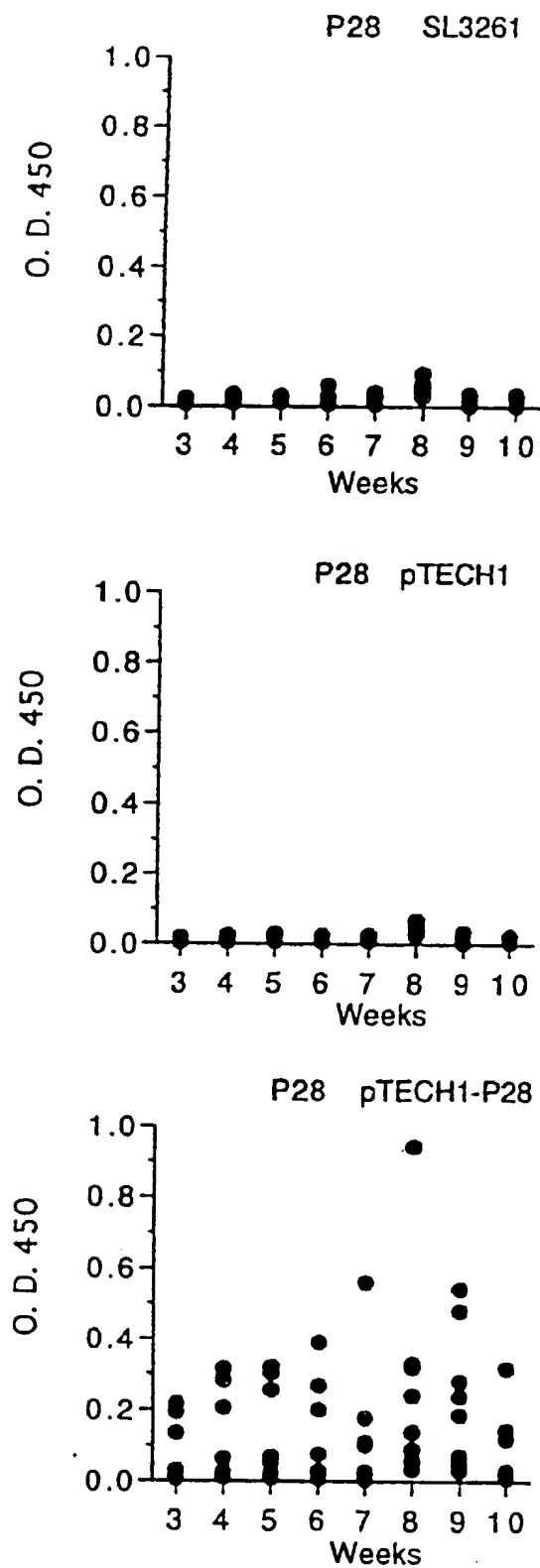


Figure 9

15/23

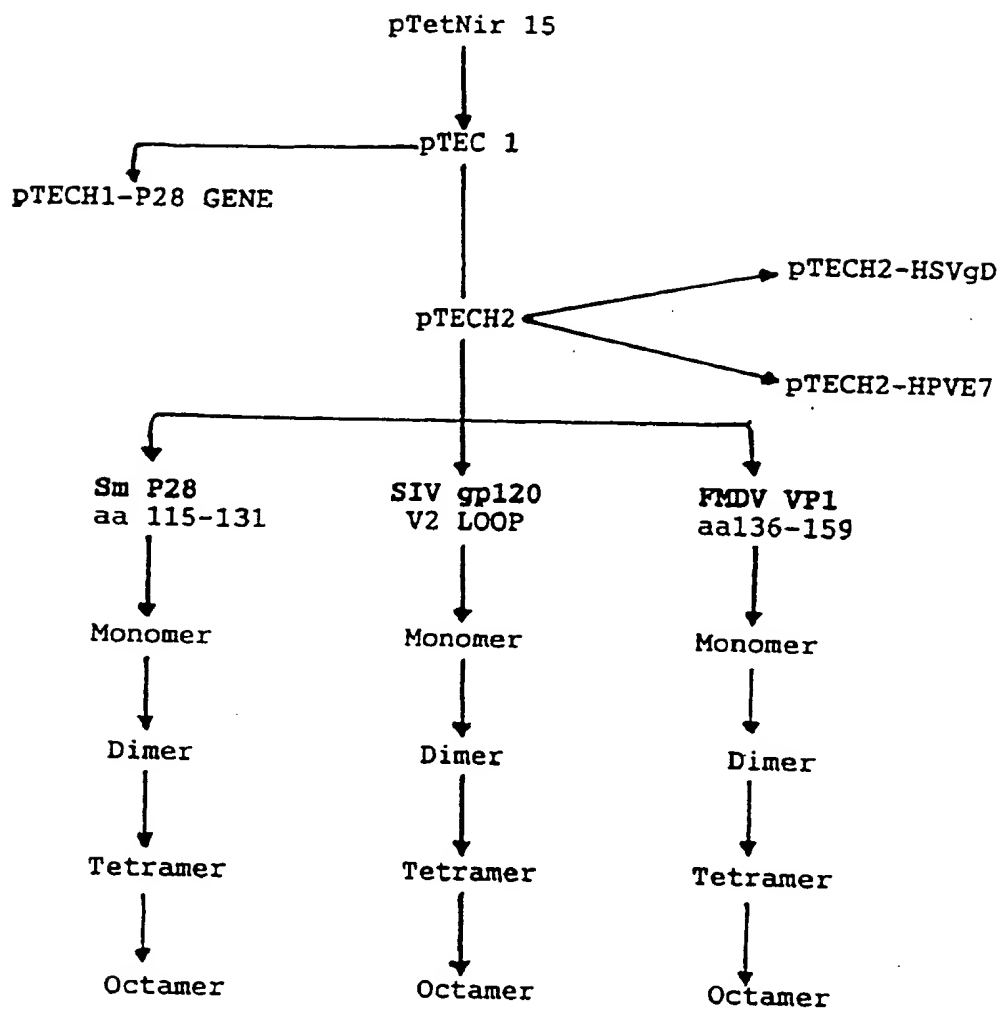
THE CONSTRUCTS

FIGURE 10

16/23

Examples of Heteromers



● = *S. mansoni* P28 epitope

▲ = SIV gp 120 V2 epitope

W = Hinge

FIGURE 11

17/23

FIGURE 12

DNA Sequence of the Vector pTECH1

(SEQ ID NO: 17)

1bp -TTCAGGTAAATTTGATGTACATCAAATGGTACCCCTTGCTGAATCGTTAAGGTAGGCGGT - 60bp
AGGGCCAGATCTTAATCATCCACAGGAGACTTTCTGATGAAAAACCTTGATTGTGGGT
CGACAACGAAGAAGACATCGATGTTATCCTGAAAAAGTCTACCATTCGAACTTGGACAT
CAACAACGATATTATCTCCGACATCTCTGGTTTCAACTCCTCTGTTATCACAATCCAGA
TGCTCAATTGGTGC CGGGCATCAACGGCAAAGCTATCCACCTGGTTAACAACGAATCTTC
TGAAGTTATCGTGACAAGGCCATGGACATCGAATACAACGACATGTTCAACAACCTCAC
CGTAGCTTCGGCTGCGCGTTCCGAAAGTTTCTGCTTCCACCTGGAACAGTAGGGCAC
TAACGAGTACTCCATCATCAGTCTATGAAGAAACACTCCCTGTCCATCGGCTCTGGTTG
GTCTGTTTCCCTGAAGGGTAACAACCTGATCTGGACTCTGAAAGACTCCGCGGGCGAAGT
TCGTCAGATCACTTTCCGCGACCTGCCGGACAAGTTCAACGGTACCTGGCTAACAAATG
GGTTTTATCACTATCACTAACGATCGTCTGTCTTCTGCTAACCTGTACATCAACGGCGT
TCTGATGGGCTCCGCTGAATCACTGGTCTGGGCGCTATCCGTGAGGACAACAACATCAC
TCTTAAGCTGGACCGTTGCAACAACAACAACAGTACGTATCCATCGACAAGTTCGGTAT
CTTCTGCAAAGCACTGAACCCGAAAGAGATCGAAAACTGTATACCAGCTACCTGTCTAT
CACCTTCTGCGTGAATTTCTGGGGTAACCCGCTGCGTTACGACACCGAATATTACCTGAT
CCCGGTAGCTTCTAGCTCTAAAGACGTTACGCTGAAAAACATCACTGACTACATGTACCT
GACCAACGGCGCGTCCCTACACTAACGGTAAACTGAACATCTACTACCGACGTCTGTACAA
CGGCCTGAAATTTCATCATCAACGCTACACTCCGAACAACGAAATCGATTCTTTCTGTTAA
ATCTGGTGAATTCATCAAACTGTACGTTCTTACAACAACAACGAACACATCGTTGGTTA
CCCGAAAGACGGTAACGCTTTCAACAACCTGGACAGAATTCTGGCTGTTGGTTACAAACGC
TCCGGGTATCCCGCTGTACAAAAAATGGAAGCTGTTAAACTGCGTGACCTGAAAACCTA
CTCTGTTACGCTGAAACTGTACGACGACAAAAACGCTTCTCTGGTCTGGTTGGTACCCA
CAACGGTCAGATCGGTAAACGACCGGAACCGTGACATCCTGATCGCTTCTAACTGGTACTT
CAACCACCTGAAAGACAAAATCCCTGGGTGCGACTGGTACTTCGTTCCGACCGATGAAGG
TTGGACCAACGAACGGGCCGGGGCCCTCTAGAATCACTAGTTAAGGATCCGCTAGCCCGCC

SUBSTITUTE SHEET

18/23

pTECH1 DNA Sequence continued

TAATGAGCGGGCTTTTTTCTCGGGCAGCGTTGGGTCTGGCCACGGGTGCGCATGATC
GTGCTCCTGTCTTGAGGACCCGGCTAGGCTGGCGGGGTTCCTTACTGGTTAGCAGAAT
GAATCACCGATACGCGAGCGAACGTGAAGCGACTGCTGCTGCAAAACGTCTGCGACCTGA
GCAACAACATGAATGGTCTTCGGTTTCCGTGTTTCGTAAAGTCTGGAAACGCGGAAGTCA
GCGCTCTTCCGCTTCCTCGCTCACTGACTCGCTCGCTCGGTCTGCTGGCTGCGGCGAGC
GGTATCAGCTCACTCAAAGGCGGTAAACGGTTATCCACAGAATCAGGGGATAACGCAGG
AAAGAACATGTGAGCAAAAGGCCAGCAAAAGGCCAGGAACCGTAAAAAGGCCGCTTGCT
GGCGTTTTTCCATAGGCTCCGCCCCCTGACGAGCATCACAAAAATCGACGCTCAAGTCA
GAGGTGGCGAAACCGACAGGACTATAAAGATACCAGGCGTTTCCCCCTGGAAGCTCCCT
CGTGGCTCTCCTGTTCGACCCCTGCGGCTTACCGGATACCTGTCCGCTTTCTCCCTTC
GGGAAGCGTGGGCTTTCTCAATGCTCAGGCTGTAGGTATCTCAGTTCCGTGTAGGTCTG
TCGCTCCAAGCTGGGCTGTGTGCAGAACCCCCCGTTCAGCCCGACCGCTGCGCCTTATC
CGGTAACATATGCTCTTGAAGTCCAACCCGGTAAGACACGACTTATCGCCACTGGCAGCAGC
CACTGGTAACAGGATTAGCAGAGCGAGGTATGTAGGCGGTGCTACAGAGTTCTTGAAGTG
GTGGCCTAACTACGGCTACACTAGAAGGACAGTATTTGGTATCTGCGCTCTGCTGAAGCC
AGTTACCTTCGGAAAAAGAGTTGGTAGCTCTTGATCCGGCAAACAAACCACCGCTGGTAG
CGGTGGTTTTTTTGTGTTGCAAGCAGCAGATTACGCGCAGAAAAAAGGATCTCAAGAAGA
TCCTTTGATCTTTTCTACGGGGTCTGACGCTCAGTGGAAACGAAACTCAGTTAAGGGAT
TTTGGTCATGAGATTATCAAAAAGGATCTTCACCTAGATCCTTTTAAATTAAAAATGAAG
TTTTAAATCAATCTAAAGTATATATGAGTAAACTTGGTCTGACAGTTACCAATGCTTAAT
CAGTGAGGCACCTATCTCAGCGATCTGTCTATTTGTTTCATCCATAGTTGCCTGACTCCC
CGTCTGTGTAGATAACTACGATACGGGAGGGCTTACCATCTGGCCCCAGTCTGCAATGAT
ACCGCGAGACCCACGCTCACCGGCTCCAGATTTATCAGCAATAAACCAGCCAGCCGGAAG
GGCCGAGCGCAGAAGTGGTCTGCAACTTTATCCGCTCCATCCAGTCTATTAATTGTTG

SUBSTITUTE SHEET

19/23

pTECH1 DNA Sequence continued

CCGGGAAGCTAGAGTAAGTAGTTGCCAGTTAATAGTTTGGCAACGTTGTGCCATTGC
TGCAGGCATCGTGGTGTACGCTCGTTCGTTGGTATGGCTTCATTACAGCTCCGGTTCCCA
ACGATCAAGGCGAGTTACATGATCCOCCATGTTGTGCAAAAAAGCGTTAGCTCCTTCGG
TCTTCGATCGTTGTGAGAAGTAAGTTGGCCGCGAGTGTATCACTCATGGTTATGGCAGC
ACTGCATAATTCTCTTACTGTCTATGCCATCCGTAAGATGCTTTTCTGTGACTGGTGAGTA
CTCAACCAAGTCATTCTGAGAATAGTGTATGCGGCGACCGAGTTGCTCTTGCCCGGCGTC
AACACGGGATAATACCGCGCCACATAGCAGAACTTTAAAAGTCTCATCATGGAAAACG
TTCTTCGGGGCGAAAACCTCTCAAGGATCTTACCGCTGTTGAGATCCAGTTGATGTAACC
CACTCGTGCACCCAACTGATCTTCAGCATCTTTTACTTTTACCAGCGTTTCTGGGTGAGC
AAAAACAGGAAGGCAAAATGCCGCAAAAAAGGGAATAAGGGCGACACGGAAATGTTGAAT
ACTCATACTCTTCCCTTTTCAATATTATTGAAGCATTATCAGGGTTATTGTCTCATGAG
CGGATACATATTTGAATGTATTTAGAAAAATAAACAAATAGGGGTTCCGCGCACATTTCC
CCGAAAAGTGCCACCTGACGTCTAAGAAACCATTATTATCATGACATTAACTATAAAAA
TAGGCGTATCACGAGGCCCTTTCTGTCCTTCAAGAA - 3754bp

SUBSTITUTE SHEET

FIGURE 13

DNA Sequence of the Vector pTECH2

(SEQ ID NO: 18)

1bp - TTCAGGTAAATTTGATGTACATCAAATGGTACCCCTTGCCTGAATCGTTAAGGTAGGCGGT - 60bp
AGGGCCCAGATCTTAATCATCCACAGGAGACTTTCTGATGAAAAACCTTGATTGTGGGT
CGACAACGAAGAAGACATCGATGTTATCCTGAAAAAGTCTACCATTCGAACTTGGACAT
CAACAACGATATATCTCOGACATCTCTGGTTTCAACTCCTCTGTTATCACAATATCCAGA
TGCTCAATTGGTGGCGGGCATCAACGGCAAGCTATCCACCTGGTAAACAACGAATCTTC
TGAAGTATCGTGCAAGGOCATGGACATCGAATAACAACGACATGTTCAACAACCTTAC
CGTTAGCTTCGGCTGGCGTTCCGAAAGTTCTGCTTCCCACTGGAAACAGTACGGCAC
TAACGAGTATCCATCATCAGCTCTATGAAGAAACACTCCCTGTCCATCGGCTCTGGTTG
GTCTGTTTCCCTGAAGGTAACAACCTGATCTGGACTCTGAAAGACTCCGCGGGCGAAGT
TCGTGAGATCACTTCCGGACCTGCGGACAAGTTCAACCGGTACCTGGCTAACAATG
GGTTTTCATCACTATCACTAACGATCGTCTGTTCTCTGCTAACCTGTACATCAACGGCGT
TCTGATGGGCTCCGCTGAAATCACTGGTCTGGGCGCTATCCGTGAGGACAACAACATCAC
TCTTAAGCTGGACCGTTGCAACAACAACCAACAGTACGTATCCATCGACAAGTTCCGTAT
CTTCTGCAAAGCACTGAACCCGAAAGAGATCGAAAACTGTATACCAGCTACCTGTCTAT
CACCTTCCTGCGTGAATCTCTGGGGTAACCCGCTGCGTTACGACACCGAATATTACCTGAT
CCCGGTAGCTTCTAGCTCTAAAGACGTTCACTGAAAAACATCACTGACTACATGTACCT
GACCAACCGCGCGTCTTACACTAACGTTAACTGAACATCTACTACCGACGTTCTGTACAA
CGGCCTGAAATTCATCATCAAACGCTACACTCCGAACAACGAATCGATTCTTTCTGTTAA
ATCTGGTGACTTCACTCAAACCTGTACGTTTCTTACAACAACAACGAACACATCGTTGGTTA
CCCGAAAGACGGTAACGCTTCAACAACCTGGACAGAATTCTGCGTGTGGTTACAACGC
TCCGGGTATCCCGCTGTACAAAAAATGGAAGCTGTAAACTGCGTGACCTGAAAACCTA
CTCTGTTCACTGAACCTGTACGACGACAAAAAGCTTCTCTGGGTCTGGTTGGTACCCA
CAACGGTCAATCGGTAAACGACCGAACCGTGACATCCTGATCGCTTCTAAGTGGTACTT
CAACCACCTEAAAGACAAAAATCCTGGGTTGCGACTGGTACTTCTGTTCCGACCGATGAAGG
TTGGACCAACGACCGGGCGGGGGCCCTCTAGAGGATCCGATATCAAGCTTACTAGTTAATG
ATCCGCTAGCCCGCCTAATGAGCGGGCTTTTTTTTCTCGGGCAGCGTTGGGTCTGGCCA
CGGTGCGCATGATCGTGCTCCTGTCTTGAAGACCCGCTAGCCTGGCGGGGTTCCTT

SUBSTITUTE SHEET

pTECH2 DNA Sequence continued

ACTGGTTAGCAGAATGAATCACGATACGCGAGCGAACGTGAAGCGACTGCTGCTGCAAA
ACGTCTGCGACCTGAGCAACAACATGAATGGTCTTCGGTTTCGGTGTTCGTAAAGTCTG
GAAACGCGGAAGTCAGCGCTCTTCGGCTTCCTCGCTCACTGACTGCTGCGCTCGGTCTG
TCGGCTGCGGCGAGCGGTATCAGCTCACTCAAAGGCGGTAATACGGTTATCCACAGAATC
AGGGGATAACGCAGGAAAGAACATGTGAGCAAAAGGCCAGCAAAAGGCCAGGAACCGTAA
AAAGGCCGCGTGTCTGGCGTTTTTCCATAGGCTCCGCCCCCTGACGAGCATCAGAAAAA
TCGACGCTCAAGTCAGAGGTGGGAAACCCGACAGGACTATAAGATAACAGGCGTTTCC
CCCTGGAAGCTCCCTGCTGCGCTCTCTGTTCCGACCTGCGCTTACCGGATACCTGTC
CGCCTTTCTCCCTTCGGGAAGCGTGGCGCTTCTCAATGCTCAGCTGTAGGTATCTCAG
TTGGTGTAGGTCTGCTCCAAGCTGGGCTGTGTGCACGAACCCCCCGTTCAGCCCGA
CCGCTGCGCCTTATCCGGTAACTATCGTCTTGAGTCCAACCCGGTAAGACACGACTTATC
GCCACTGGCAGCAGCCACTGGTAACAGGATTAGCAGAGCGAGGTATGTAGGOGGTGCTAC
AGAGTTCCTGAAGTGGTGGCCTAACTACGGCTACACTAGAAGGACAGTATTTGGTATCTG
CGCTCTGCTGAAGCCAGTTACCTTCGGAAAAAGAGTTGGTAGCTCTTGATCCGGCAAACA
AACCACCGCTGGTAGCGGTGGTTTTTTTGTGTTGCAAGCAGCAGATTACGCGCAGAAAAAA
AGGATCTCAAGAAGATCCTTTGATCTTTTCTACGGGGTCTGACGCTCAGTGGAACGAAAA
CTCACGTTAAGGATTTTGGTCATGAGATTATCAAAAAGGATCTTCACCTAGATCCTTTT
AAATTAAAAATGAAGTTTTTAAATCAATCTAAAGTATATATGAGTAAACTTGGTCTGACAG
TTACCAATGCTTAATCAGTGAGGCACCTATCTCAGCGATCTGTCATTTTCTGTTTCATCCAT
AGTTGCGTGACTCCCCGTCGTGTAGATAACTACGATAACGGGAGGGCTTACCATCTGGCCC
CAGTGCTGCAATGATACCGCGAGACCCACGCTCACCGGCTCCAGATTTATCAGCAATAAA
CCAGCCAGCCGGAAGGGCCGAGCGCAGAAGTGGTCCCTGCAACTTTATCCGCTCCATCCA
GTCTATTAAATTGTTGCCGGAAGCTAGAGTAAGTAGTTGCCAGTTAATAGTTTGCGCAA
CGTTGTGCAATTGCTGCAGGCATGTTGGTGTCAAGCTCGTCTGTTGGTATGGCTTCATT
CAGCTCCGGTTCCCAACGATCAAGGCGAGTTACATGATCCCCCATGTTGTGCAAAAAAGC
GGTTAGCTCCTTCGGTCCCTCCGATCGTTGTGAGAAAGTAAGTTGGCCGAGTGTATCACT
CATGGTTATGGCAGCACTGCATAATTCTCTTACTGTGTCATGCCATCCGTAAGATGCTTTTC

SUBSTITUTE SHEET

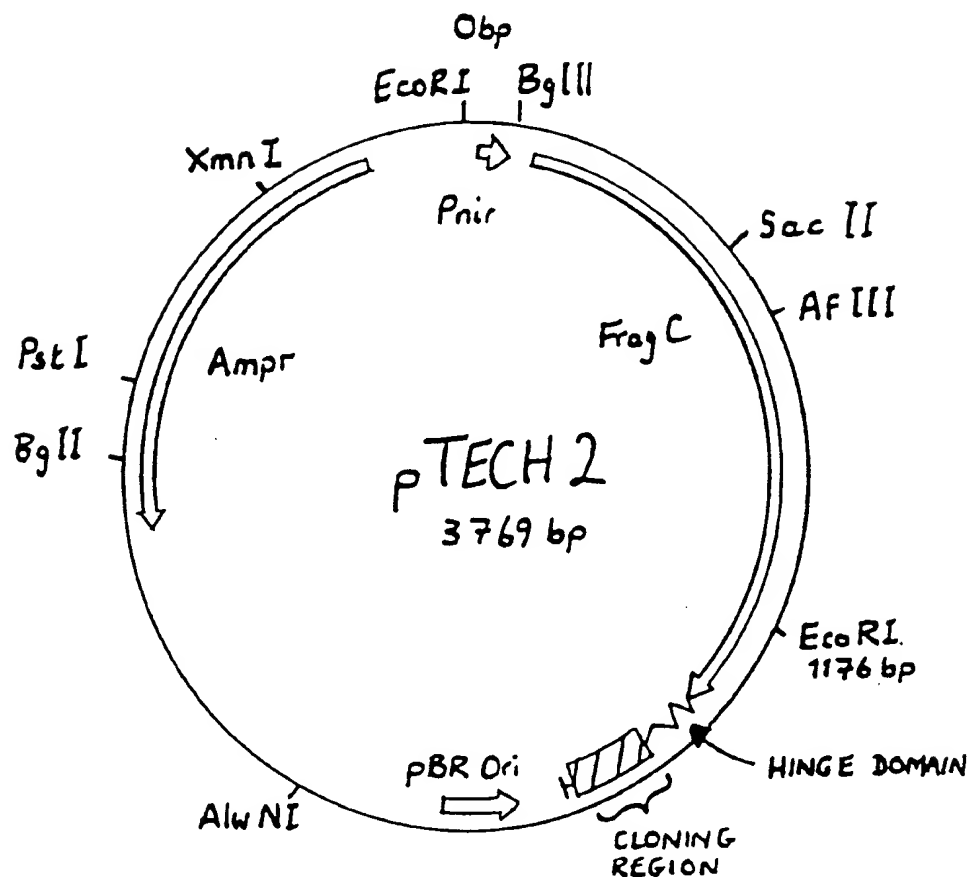
22/23

pTECH2 DNA Sequence continued

TGTGACTGGTGAGTACTCAACCAAGTCATTCTGAGAATAGTGTATGCGGCGACCGAGTTG
CTCTTGCCCGCGTCAACACGGGATAATAACGCGCCACATAGCAGAACTTTAAAAGTGCT
CATCATTTGGAAAACGTTCTTCGGGGGCGAAAACCTCTCAAGGATCTTACCGCTGTTGAGATC
CAGTTGATGTAAACCACTGTGCACCCAACTGATCTTCAGCATCTTTTACTTTTACCAG
CGTTTCTGGGTGAGCAAAAACAGGAAGGCAAAATGCGCAAAAAGGGAATAAGGGCGAC
ACGGAAATGTTGAATACTCATACTCTTCCTTTTCAATATTATTGAAGCATTATCAGGG
TTATTGTCCTCATGAGCGGATACATATTTGAATGTATTTAGAAAAATAAACAAATAGGGGT
TCCGCGCACATTTCCCGAAAAGTGCCACCTGACGTCTAAGAAACCATTATTATCATGAC
ATTAAOCTATAAAAATAGGCGTATCACGAGGCCCTTTGCTCTCAAGAA

SUBSTITUTE SHEET

23/23



	XbaI	BamHI	EcoRV	HindIII	SpeI	Stop	BamHI
---HINGE---	TCTAGA	GGATCC	GATATC	AAGCTT	ACTAGT	TAA	TGATC
	AGATCT	CCTAGG	CTATAG	TTCGAA	TGATCA	ATT	ACTAG
	(SEQ ID NO: 19)						
---GPGP----	S	R	G	S	D	I	K
	L	T	S	*			
	(SEQ ID NO: 20)						

FIGURE 14

BUDAPEST TREATY ON THE INTERNATIONAL
RECOGNITION OF THE DEPOSIT OF MICROORGANISMS
FOR THE PURPOSES OF PATENT PROCEDURE

INTERNATIONAL FORM

TO
Medeva Holdings BV
Churchill-Laan 223
Amsterdam 1078-ED

NAME AND ADDRESS
OF DEPOSITOR

RECEIPT IN THE CASE OF AN ORIGINAL DEPOSIT
issued pursuant to Rule 7.1 by the
INTERNATIONAL DEPOSITARY AUTHORITY
identified at the bottom of this page

Attention: Dr S N Chatfield
Vaccine Research Unit
Imperial College of Science & Technology
Department of Biochemistry
London SW7 2AY

I. IDENTIFICATION OF THE MICROORGANISM	
Identification reference given by the DEPOSITOR:	Accession number given by the INTERNATIONAL DEPOSITARY AUTHORITY:
Salmonella typhimurium BRD991	NCTC 12831
II. SCIENTIFIC DESCRIPTION AND/OR PROPOSED TAXONOMIC DESIGNATION	
The microorganism identified under I above was accompanied by:	
<input type="checkbox"/> a scientific description	
<input checked="" type="checkbox"/> a proposed taxonomic designation	
(Mark with a cross where applicable)	
III. RECEIPT AND ACCEPTANCE	
This International Depositary Authority accepts the microorganism identified under I above, which was received by it on 15 July 1993 (date of the original deposit) ¹	
IV. RECEIPT OF REQUEST FOR CONVERSION N/A	
The microorganism identified under I above was received by this International Depositary Authority on (date of the original deposit) and a request to convert the original deposit to a deposit under the Budapest Treaty was received by it on (date of receipt of request for conversion)	
V. INTERNATIONAL DEPOSITARY AUTHORITY	
Name: National Collection of Type Cultures Central Public Health Laboratory	Signature(s) of person(s) having the power to represent the International Depositary Authority or of authorized official(s)
Address: 61 Colindale Avenue London NW9 5HT	Date: 7 September 1993 <i>E. R. Hill</i> Curator

¹ Where Rule 6.4(d) applies, such date is the date on which the status of international depositary authority was acquired.

**BUDAPEST TREATY ON THE INTERNATIONAL
RECOGNITION OF THE DEPOSIT OF MICROORGANISMS
FOR THE PURPOSES OF PATENT PROCEDURE**

INTERNATIONAL FORM

TO
Medeva Holdings BV
Churchill-Laan 223
Amsterdam 1078-ED

NAME AND ADDRESS
OF DEPOSITOR

RECEIPT IN THE CASE OF AN ORIGINAL DEPOSIT
issued pursuant to Rule 7.1 by the
INTERNATIONAL DEPOSITARY AUTHORITY
identified at the bottom of this page

Attention: Dr S N Charfield
Vaccine Research Unit
Imperial College of Science & Technology
Department of Biochemistry
London SW7 2AY

I. IDENTIFICATION OF THE MICROORGANISM	
Identification reference given by the DEPOSITOR: Salmonella typhimurium BRD992	Accession number given by the INTERNATIONAL DEPOSITARY AUTHORITY: NCTC 12832
II. SCIENTIFIC DESCRIPTION AND/OR PROPOSED TAXONOMIC DESIGNATION	
The microorganism identified under I above was accompanied by:	
<input type="checkbox"/> a scientific description <input checked="" type="checkbox"/> a proposed taxonomic designation (Mark with a cross where applicable)	
III. RECEIPT AND ACCEPTANCE	
This International Depositary Authority accepts the microorganism identified under I above, which was received by it on 15 July 1993 (date of the original deposit) ¹	
IV. RECEIPT OF REQUEST FOR CONVERSION N/A	
The microorganism identified under I above was received by this International Depositary Authority on (date of the original deposit) and a request to convert the original deposit to a deposit under the Budapest Treaty was received by it on (date of receipt of request for conversion)	
V. INTERNATIONAL DEPOSITARY AUTHORITY	
Name: National Collection of Type Cultures Central Public Health Laboratory Address: 61 Colindale Avenue London NW9 5HT	Signature(s) of person(s) having the power to represent the International Depositary Authority or of authorized official(s) Date: 7 September 1993 <i>L.R. Hill</i> L R Hill Curator

¹ Where Rule 6.4(d) applies, such date is the date on which the status of international depositary authority was acquired.

BUDAPEST TREATY ON THE INTERNATIONAL
RECOGNITION OF THE DEPOSIT OF MICROORGANISMS
FOR THE PURPOSES OF PATENT PROCEDURE

INTERNATIONAL FORM

TO
Medeva Holdings BV
Churchill-Laan 223
Amsterdam 1078-ED

RECEIPT IN THE CASE OF AN ORIGINAL DEPOSIT
issued pursuant to Rule 7.1 by the
INTERNATIONAL DEPOSITARY AUTHORITY
identified at the bottom of this page

Attention: Dr S N Chatfield
Vaccine Research Unit
Imperial College of Science & Technology
Department of Biochemistry
London SW7 2AY

NAME AND ADDRESS
OF DEPOSITOR

I. IDENTIFICATION OF THE MICROORGANISM	
Identification reference given by the DEPOSITOR: Salmonella typhimurium BRD993	Accession number given by the INTERNATIONAL DEPOSITARY AUTHORITY: NCTC 12833
II. SCIENTIFIC DESCRIPTION AND/OR PROPOSED TAXONOMIC DESIGNATION	
The microorganism identified under I above was accompanied by:	
<input type="checkbox"/> a scientific description <input checked="" type="checkbox"/> a proposed taxonomic designation (Mark with a cross where applicable)	
III. RECEIPT AND ACCEPTANCE	
This International Depositary Authority accepts the microorganism identified under I above, which was received by it on 15 July 1993 (date of the original deposit) ¹	
IV. RECEIPT OF REQUEST FOR CONVERSION N/A	
The microorganism identified under I above was received by this International Depositary Authority on (date of the original deposit) and a request to convert the original deposit to a deposit under the Budapest Treaty was received by it on (date of receipt of request for conversion)	
V. INTERNATIONAL DEPOSITARY AUTHORITY	
Name: National Collection of Type Cultures Central Public Health Laboratory Address: 61 Colindale Avenue London NW9 5HT	Signature(s) of person(s) having the power to represent the International Depositary Authority or of authorized official(s): Date: 7 September 1993 <i>L. R. Hill</i> L R Hill Curator

¹ Where Rule 6.4(d) applies, such date is the date on which the status of international depositary authority was acquired.

**BUDAPEST TREATY ON THE INTERNATIONAL
RECOGNITION OF THE DEPOSIT OF MICROORGANISMS
FOR THE PURPOSES OF PATENT PROCEDURE**

INTERNATIONAL FORM

TO
Medeva Holdings BV
Churchill-Laan 223
Amsterdam 1078-ED

RECEIPT IN THE CASE OF AN ORIGINAL DEPOSIT
issued pursuant to Rule 7.1 by the
INTERNATIONAL DEPOSITARY AUTHORITY
identified at the bottom of this page

NAME AND ADDRESS
OF DEPOSITOR

Attention: Dr S N Chatfield
Vaccine Research Unit
Imperial College of Science & Technology
Department of Biochemistry
London SW7 2AY

I. IDENTIFICATION OF THE MICROORGANISM	
Identification reference given by the DEPOSITOR: Salmonella typhimurium BRD994	Accession number given by the INTERNATIONAL DEPOSITARY AUTHORITY: NCTC 12834
II. SCIENTIFIC DESCRIPTION AND/OR PROPOSED TAXONOMIC DESIGNATION	
The microorganism identified under I above was accompanied by:	
<input type="checkbox"/> a scientific description	
<input checked="" type="checkbox"/> a proposed taxonomic designation	
(Mark with a cross where applicable)	
III. RECEIPT AND ACCEPTANCE	
This International Depositary Authority accepts the microorganism identified under I above, which was received by it on 15 July 1993 (date of the original deposit) ¹	
IV. RECEIPT OF REQUEST FOR CONVERSION N/A	
The microorganism identified under I above was received by this International Depositary Authority on (date of the original deposit) and a request to convert the original deposit to a deposit under the Budapest Treaty was received by it on (date of receipt of request for conversion)	
V. INTERNATIONAL DEPOSITARY AUTHORITY	
Name: National Collection of type Cultures Central Public Health Laboratory	Signature(s) of person(s) having the power to represent the International Depositary Authority or of authorized official(s): Date: 7 September 1993 <i>C. R. Hill</i> Curator
Address: 61 Colindale Avenue London NW9 5HT	

¹ Where Rule 6.4(d) applies, such date is the date on which the status of international depositary authority was acquired.

BUDAPEST TREATY ON THE INTERNATIONAL
RECOGNITION OF THE DEPOSIT OF MICROORGANISMS
FOR THE PURPOSES OF PATENT PROCEDURE

INTERNATIONAL FORM

TO
Medeva Holdings BV
Churchill-Laan 223
Amsterdam 1078-ED

NAME AND ADDRESS
OF DEPOSITOR

RECEIPT IN THE CASE OF AN ORIGINAL DEPOSIT
issued pursuant to Rule 7.1 by the
INTERNATIONAL DEPOSITARY AUTHORITY
identified at the bottom of this page

Attention: Dr S N Chatfield
Vaccine Research Unit
Imperial College of Science & Technology
Department of Biochemistry
London SW7 2AY

I. IDENTIFICATION OF THE MICROORGANISM	
Identification reference given by the DEPOSITOR: Escherichia coli BRD970	Accession number given by the INTERNATIONAL DEPOSITARY AUTHORITY: NCTC 12837
II. SCIENTIFIC DESCRIPTION AND/OR PROPOSED TAXONOMIC DESIGNATION	
The microorganism identified under I above was accompanied by:	
<input type="checkbox"/> a scientific description <input checked="" type="checkbox"/> a proposed taxonomic designation (Mark with a cross where applicable)	
III. RECEIPT AND ACCEPTANCE	
This International Depositary Authority accepts the microorganism identified under I above, which was received by it on 23 July 1993 (date of the original deposit) ¹	
IV. RECEIPT OF REQUEST FOR CONVERSION N/A	
The microorganism identified under I above was received by this International Depositary Authority on (date of the original deposit) and a request to convert the original deposit to a deposit under the Budapest Treaty was received by it on (date of receipt of request for conversion)	
V. INTERNATIONAL DEPOSITARY AUTHORITY	
Name: National Collection of Type Cultures Central Public Health Laboratory Address: 61 Colindale Avenue London NW9 5HT	Signature(s) of person(s) having the power to represent the International Depositary Authority or of authorized official(s): Date: 7 September 1993 <i>L.R. Hill</i> L R Hill Curator

¹ Where Rule 6.4(d) applies, such date is the date on which the status of international depositary authority was acquired.

INTERNATIONAL SEARCH REPORT

International Application No

PCT/GB 93/01617

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) ⁶		
According to International Patent Classification (IPC) or to both National Classification and IPC		
Int.Cl. 5 C12N15/62; C12N15/31; C12N15/54; C12N1/21 C07K13/00; A61K39/08; //(C12N1/21, C12R1:42)		
II. FIELDS SEARCHED		
Minimum Documentation Searched ⁷		
Classification System	Classification Symbols	
Int.Cl. 5	C12N ; C07K ; A61K	
Documentation Searched other than Minimum Documentation to the extent that such documents are included in the fields searched ⁸		
III. DOCUMENTS CONSIDERED TO BE RELEVANT⁹		
Category ¹⁰	Citation of Document, ¹¹ with indication, where appropriate, of the relevant passages ¹²	Relevant to Claim No. ¹³
Y	NATURE vol. 330, 12 November 1987, MACMILLAN JOURNALS LTD., LONDON, UK; pages 168 - 170 M.J. FRANCIS ET AL. 'Non-responsiveness to a foot-and-mouth disease virus peptide overcome by addition of foreign helper T-cell determinants' see page 168, left column, line 1 - page 170, right column, line 18 ---	1-24
Y	WO,A,8 906 974 (PRAXIS BIOLOGICS, INC.) 10 August 1989 see page 25, line 12 - page 26, line 9 see page 26, line 25 - page 27, line 14 see page 76, line 1 - page 81, line 4; claims 1-107; tables 12-15 --- <div style="text-align: right;">-/--</div>	1-24

¹⁰ Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with

INTERNATIONAL SEARCH REPORT

International Application No

PCT/GB 93/01617

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)		
Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
Y	EP,A,0 432 965 (SMITHKLINE BEECHAM) 19 June 1991 see page 16, line 24 - line 29 ---	1-24
Y	NUCLEIC ACIDS RESEARCH vol. 19, no. 11, 11 June 1991, IRL, OXFORD UNIVERSITY PRESS, UK; pages 2889 - 2892 M.D. OXER ET AL. 'High level heterologous expression in E. coli using the anaerobically-activated nirB promoter' cited in the application see page 2890, left column, paragraph 4 - page 2892, right column, line 19 ---	1-24
Y	WO,A,9 109 621 (INSTITUT PASTEUR) 11 July 1991 see page 2, line 12 - line 15 ---	1-24
Y	J. IMMUNOLOGY vol. 141, no. 5, 1 September 1988, AM. SOC. IMMUNOLOGISTS, US; pages 1687 - 1694 C. AURIAULT ET AL. 'Analysis of T and B cell epitopes of the Schistosoma mansoni P28 antigen in the rat model by using synthetic peptides' see page 1688, left column, line 18 - line 29 ---	1-24
P, Y	WO,A,9 215 689 (THE WELLCOME FOUNDATION LIMITED) 17 September 1992	1-24

ANNEX TO THE INTERNATIONAL SEARCH REPORT
ON INTERNATIONAL PATENT APPLICATION NO.

GB 9301617
SA 77553

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report.
The members are as contained in the European Patent Office EDP file on
The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

14/10/93

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO-A-8906974	10-08-89	AU-B- 634153	18-02-93
		AU-A- 3065489	25-08-89
		EP-A- 0399001	28-11-90
		JP-T- 3502691	20-06-91
EP-A-0432965	19-06-91	AU-B- 634837	04-03-93
		AU-A- 6777490	13-06-91
		CA-A- 2031468	09-06-91
		CN-A- 1053814	14-08-91
WO-A-9109621	11-07-91	FR-A- 2656626	05-07-91
WO-A-9215689	17-09-92	AU-A- 1350892	06-10-92
		WO-A- 9215688	17-09-92